



Physico-chemical properties, oxidative stability and non-enzymatic browning in marine phospholipid emulsions and their use in food applications

Lu, Henna Fung Sieng; Jacobsen, Charlotte; Baron, Caroline P.; Nielsen, Nina Skall

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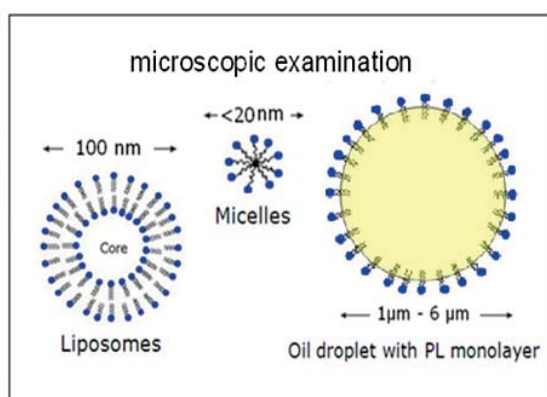
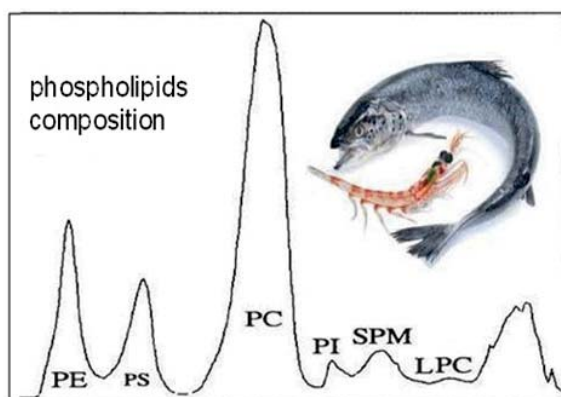
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Physico-chemical properties, oxidative stability and non-enzymatic browning in marine phospholipid emulsions and their use in food applications



Henna Fung Sieng Lu
PhD Thesis
2013

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**Henna Lu Fung Sieng
Ph.D. Thesis
2013**

**Division of Industrial Food Research
National Food Institute
Technical University of Denmark**

PREFACE

The present Ph.D. thesis entitled: “Physico-chemical properties, oxidative stability and non-enzymatic browning in marine phospholipids and their use in food applications” is submitted as a part of the requirements for obtaining a Ph.D. degree at Technical University of Denmark.

The present Ph.D. study was conducted at Division of Industrial Food Research, National Food Institute from 15th October 2009 to 14th October 2012 (3 years) under supervision of Professor Charlotte Jacobsen (main supervisor) and two senior research scientists, Dr. Nina Skall Nielsen and Dr. Caroline Baron as co-supervisors.

During this Ph.D. study, I was away for one week research at Spectra Service GmbH, Cologne, Germany in July 2011 to learn phospholipids isolation by using column chromatography and the measurements of phospholipid composition by using ¹³C NMR and P NMR techniques.

The present Ph.D. study was a part of “Healthy Growth” project and was partly financed by Øresund Food Network (ØFN). Alfa Laval and Triple Nine are collaborators of this Ph.D. study. Some of the commercial marine phospholipids used in this Ph.D. study were supplied by Triple Nine. Collaborators also participated in this Ph.D. study as external scientific advisor.

In addition, I was selected as a recipient of an AOCS Honored Student Award recently. This award will partially support my participation at 104th AOCS Annual Meeting & Expo in Montreal, Quebec, Canada, April 28- May 1, 2013.

January 14, 2013

Kgs. Lyngby, Denmark

Henna Lu Fung Sieng

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With sincere gratitude, I also would like to thank all lab technicians (Lis Berner, Inge Holmberg, Victoria Rothman and Thi Thu Trang Vu) for their technical help and guidance in the lab and Birgitte Raagaard Thomsen (M.Sc. student) for her experimental work.

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- a) Hans Otto - for supplying marine phospholipids and arranging a visit to his marine phospholipids manufacturing plant.
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- d) Dr. Bernd Diehl – for the help in analyzing marine phospholipids composition by using P NMR.
- e) Assoc. Prof. Huiling Mu – for the help in measuring zeta potential of marine phospholipids emulsion.

Last but not least, I would like to thank my parents, close friends and colleagues for their prayers, love, care and moral support.

SUMMARY

Marine phospholipids (PL) contain a high level of eicosapentaenoic acids (EPA) and docosahexaenoic acids (DHA), which have documented beneficial effect on human health. In addition, marine PL are more advantageous than crude or refined fish oils. Marine PL are more resistant to oxidation, provide better bioavailability and ability to form liposomes. All these unique properties of marine PL make them an attractive choice as ingredients for food fortification. Nowadays, a wide range of food products fortified with n-3 triglycerides (TAG) are available worldwide. However, the feasibility of using marine PL for food fortification has not been explored. The main objective of the present Ph.D. study was to explore the feasibility of using marine PL for food fortification. The secondary objective was to study the physical and oxidative stability of marine PL emulsions while identifying the important factors affecting their stability.

Marine PL contain a high level of phosphatidylcholine (PC), which has amphiphilic properties. Therefore it is feasible to prepare marine PL emulsions without addition of other emulsifiers. Emulsions containing solely marine PL with a high physical stability could be prepared by using 2-10 % marine PL. The high physical stability of these emulsions was most likely due to the coexistence of micelles, liposomes and emulsified oil droplets. However, there was a requirement for at least 3 % of marine PL (equivalent to 0.8 - 1.3 % of PC depending on the marine PL sources) to avoid phase separation and to form physically stable emulsions containing both marine PL and fish oil.

Emulsions with high oxidative stability could be prepared by using marine PL of high quality with a high content of PL, cholesterol, antioxidants and a low content of prooxidants such as transition metals and initial hydroperoxides. In addition, the presence of other antioxidative compounds such as residues of free amino acids and pyrroles (formed via non-enzymatic browning reactions) in marine PL most likely have improved the oxidative stability of marine PL emulsions. In addition, hydrolysis of PL in marine PL emulsions was minimal at pH 7. In general, both physical and oxidative stability of marine PL emulsions varied in relation to the chemical composition of the marine PL used for emulsion preparation. Therefore, marine PL were purified through acetone precipitation in order to eliminate the effect of other factors such as the content of TAG, antioxidant or other minor components on lipid oxidation in marine PL. The oxidative stability of emulsions prepared from different levels of purified marine PL was investigated. Results obtained seem to

suggest that the oxidative stability of purified marine PL emulsions was greatly improved by the addition of α -tocopherol.

Non-enzymatic browning reactions were observed in marine PL emulsions through the a) measurements of Strecker degradation (SD) products of amino acid residues, and b) measurements of hydrophobic and hydrophilic pyrroles (which are pyrrolisation products of phosphatidylethanolamine (PE) and amino acids), respectively. Several mechanisms were proposed for non-enzymatic browning reactions in marine PL. It is speculated that these reactions might have occurred in marine PL mainly during their manufacturing process due to the interactions between lipid oxidation products with the primary amine groups from PE and residues of amino acids/protein that are present in marine PL. In addition, the content of pyrroles, SD products and the degree of browning in marine PL might be influenced by chemical compositions of marine PL and their manufacturing processes. In order to further investigate if the presence of pyrroles or degradation products of amino acids have any influence on oxidative stability of marine PL, liposomal dispersions were prepared from pure PC and PE compounds and purified marine PL with and without addition of amino acids. The obtained result from this model study confirmed the proposed mechanisms of non-enzymatic browning reactions in marine PL. The presence of PE and amino acids led to formation of pyrroles, generation of SD products and decreases in both browning development and lipid oxidation in liposomal dispersions. The low lipid oxidation in dispersions containing amino acids might be attributed to the antioxidative properties of pyrroles or amino acids. In addition, it is speculated that PE and amino acids pyrrolisation or oxypolymerisation of lipid oxidation products in marine PL might be the cause of browning development.

Incorporation of marine PL into fermented milk product adversely affected the oxidative stability and sensory quality of fortified products despite the use of a low percentage of marine PL in combination with fish oil for fortification. This unexpected result was mainly due to the quality of current marine PL that was used for emulsion preparation and food application. In addition, the oxidative stability and sensory quality of marine PL fortified products varied depending on the quality and source of marine PL used for fortification. Although the attempts to incorporate marine PL into food system did not produce the expected results, the findings from the present Ph.D. study provide food industries and academia with new insights into the oxidative stability of marine PL and further inspirations for improving the quality of current marine PL.

SAMMENFATNING

Marine phospholipider (PL) har et højt indhold af eicosapentaensyre (EPA) og docosahexaensyre (DHA), som har en dokumenteret sundhedsfremmende effekt på mennesker. Udover den gavnlige effekt fra EPA og DHA har marine PL også andre fordele, som rå og raffinerede fiskeolier ikke har. Marine PL er mere modstandsdygtige overfor oxidation, de er mere biotilgængelige og har amphiphile egenskaber samt evnen til at danne liposomer. Alle disse unikke egenskaber gør marine PL til en attraktiv ingrediens til fødevarerberigelse. I dag findes der en bred vifte af fødevarer beriget med n-3 triglycerider (TAG) over hele verdenen. Dog er anvendeligheden af marine PL til fødevarerberigelse ikke blevet udforsket. Hovedformålet med dette Ph.D. studium var at undersøge mulighederne for at anvende marine PL til fødevarerberigelse. Det sekundære formål var at studere den fysiske og oxidative stabilitet af marine PL emulsioner og derved identificere vigtige faktorer, som kan påvirke deres stabilitet.

Marine PL har et højt indhold af phosphatidylcholin (PC), som har amphiphile egenskaber. Det var derfor muligt at fremstille marine PL emulsioner uden tilsætning af andre emulgatorer. Emulsioner, kun emulgeret af marine PL, med en høj fysisk stabilitet kunne fremstilles, når der blev tilsat 2-10 % marine PL. Den høje fysiske stabilitet af disse emulsioner skyldes sandsynligvis sameksistens af miceller, liposomer og emulgerede oliedråber. For at danne en fysisk stabil emulsion indeholdende både marine PL og fiskeolie kræves der dog mindst 3 % marine PL (svarende til 0,8-1,3 % PC afhængig af typen af marine PL). Emulsioner med en høj oxidativ stabilitet kunne fremstilles ved brug af marine PL af høj kvalitet med et højt indhold af PL, kolesterol, antioxidanter og et lavt indhold af prooxidanter såsom overgangsmetaller og allerede eksisterende hydroperoxider. Desuden kan tilstedeværelsen af andre antioxidative forbindelser, såsom frie aminosyrer og pyrroler (dannet via ikke-enzymatiske bruningsreaktioner), i marine PL højest sandsynligt forbedre den oxidative stabilitet af marine PL emulsioner. Desuden var hydrolysen af PL i marine PL emulsioner minimal ved pH 7. Generelt varierede både den fysiske og den oxidative stabilitet af marine PL emulsioner afhængig af den kemiske komposition af marine PL, som blev brugt til fremstilling af emulsionen. Derfor blev marine PL oprenset via acetone præcipitation med henblik på at eliminere effekten på lipid oxidation i marine PL af andre faktorer, såsom indholdet af TAG, antioxidanter eller andre mindre komponenter. Den oxidative stabilitet af emulsioner fremstillet af marine PL med forskellige oprensingsniveauer blev undersøgt. De

opnåede resultater indikerede, at den oxidative stabilitet af de oprensede marine PL emulsioner blev væsentligt forbedret ved tilsætning af α -tocopherol.

Ikke-enzymatiske bruningsreaktioner blev observeret i marine PL emulsioner via henholdsvis a) målinger af streckers nedbrydningsprodukter (som er nedbrydningsprodukter af aminosyrer) og b) målinger af hydrofobe og hydrofile pyrroler (som er pyrrolisationsprodukter af phosphatidylethanolamin (PE) og aminosyrer). Forskellige mekanismer blev foreslået for ikke-enzymatiske bruningsreaktioner i marine PL. Det er sandsynligt, at disse reaktioner hovedsagligt sker under produktionen af marine PL som følge af reaktioner imellem lipidoxidationsprodukter med primært aminogruppen fra PE og rester af aminosyrer/proteiner, der er til stede i marine PL. Derudover kan indholdet af pyrroler, streckers nedbrydningsprodukter og bruningsgraden af marine PL blive påvirket af den kemiske komposition og produktionsmetoden af marine PL. Med henblik på at undersøge om tilstedeværelsen af pyrroler eller nedbrydningsprodukter fra aminosyrer havde en indflydelse på den oxidative stabilitet af marine PL blev liposomale dispersioner fremstillet af rene PC og PE forbindelser og oprensede marine PL med og uden tilsætning af aminosyrer. De opnåede resultater fra denne modelundersøgelse bekræftede den foreslåede mekanisme for ikke-enzymatiske bruningsreaktioner i marine PL. Tilstedeværelsen af PE og aminosyrer førte til dannelse af pyrroler, generering af streckers nedbrydningsprodukter og reduktion af både bruningsfarvningen og lipid oxidation i liposomale dispersioner. Den lave grad af lipid oxidation i dispersioner indeholdende aminosyrer kan muligvis tilskrives de antioxidative egenskaber af pyrroler eller aminosyrer. Desuden er det muligt, at PE- og aminosyre-pyrrolisering eller oxypolymerisation af lipid oxidationsprodukter i marine PL kan forårsage bruningen. Inkorporering af marine PL i et fermenteret mælkeprodukt påvirkede i høj grad den oxidative stabilitet og den sensoriske kvalitet af det berigede fermenterede mælkeprodukt på trods af, at der blev anvendt et lavt procentvis indhold af marine PL i kombination med fiskeolie til berigelse af det fermenterede mælkeprodukt. Dette uventede resultat skyldtes hovedsagligt kvaliteten af de marine PL, som blev brugt til fremstillingen af emulsioner og fødevarerberigelsen. Derudover varierede den oxidative stabilitet og den sensoriske kvalitet afhængig af kvaliteten og kilden af marine PL anvendt til berigelsen. Selvom forsøget på at inkorporer marine PL i fødevarer systemer ikke resulterede i det forventede resultat, kan resultaterne fra dette Ph.D. studie bidrage til, at fødevarerindustrien og den akademiske verden får en ny indsigt i den oxidative stabilitet af marine PL og derudover inspirere til at forbedre kvaliteten af de nuværende marine PL.

ABBREVIATIONS

A•	antioxidant radical
AH	primary antioxidant
BHA	butylhydroquinone
BHT	butylated hydroxytoluene
CL	cardiolipin
EPA	eicosapentaenoic acid
DHA	docosahexaenoic acid
DHS	dynamic headspace analysis
DIM	dimer
DPC	dispersion prepared from pure phosphatidylcholine
DPCA	dispersion prepared from pure phosphatidylcholine with amino acids added
DPE	dispersion prepared from phosphatidylethanolamine
DPEA	dispersion prepared from phosphatidylethanolamine with amino acids added
DLC	dispersion prepared from purified LC
DLCA	dispersion prepared from purified LC with amino acids added
DMPW	dispersion prepared from purified MPW
DMPWA	dispersion prepared from purified MPW with amino acids added
ES	emulsion separation
GC-MS	gas chromatography mass spectrometry
H•	hydrogen radical
L•	lipid radical
LC	marine phospholipids received from PhosphoTech
LO•	alkoxy radical
LOO•	peroxy radical
LH	unsaturated lipid
LOOH	lipid peroxide
LPC	lysophosphatidylcholine
Lys	lysine
MGK	marine phospholipids received from Polaris
MPL	marine phospholipids with ethoxyquin added, received from Triple Nine
MPT	marine phospholipids received from University of Tromsø
MPN	marine phospholipids with an improved quality, received from Triple Nine
MPW	marine phospholipids without ethoxyquin, received from Triple Nine
•OH	hydroxyl
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	propyl gallate
PI	phosphatidylinositol
PS	phosphatidylserine
PSD	particle size distribution
PL	glycerophospholipids
LC PUFA	long chain polyunsaturated fatty acid
PV	peroxide value
SD	strecker degradation
SPM	sphingomyelin
SPME	solid phase micro-extraction
TAG	triglycerides

TBARS	thiobarbituric reactive substances
TBHQ	tertiary butylhydroquinone
TET	tetramers
TRI	trimer
TL	total lipid

LIST OF PUBLICATIONS

- I. Lu, F. S. H., Nielsen, N. S., Heinrich, M. T., Jacobsen, C. (2011). Oxidative stability of marine phospholipids in the liposomal form and their applications: A review. *Lipids*, 46, 3-23.
- II. Lu, F. S. H., Nielsen, N. S., Baron, C. P., Jensen, L. H. S., & Jacobsen, C. (2012). Physico-chemical properties of marine phospholipid emulsions. *Journal of the American Oil Chemists' Society*, 89, 2011-2024.
- III. Lu, F. S. H., Nielsen, N. S., Baron, C. P., & Jacobsen, C. (2012). Oxidative degradation and non-enzymatic browning due to the interaction between oxidized lipids and primary amine groups in different marine phospholipid emulsions. *Food Chemistry*, 135, 2887-2896.
- IV. Lu, F. S. H., Nielsen, N. S., Baron, C. P., Diehl, B. W. K., & Jacobsen, C. (2012). Oxidative stability of emulsions prepared from purified marine phospholipid and the role of α -tocopherol. *Journal of Agricultural and Food Chemistry*, 60, 12388-12396.
- V. 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 phospholipid stability: Non-enzymatic browning and lipid oxidation. *Food Chemistry*, 141, 879-888.
- VI. Lu, F. S. H., Thomsen, B. R., Hyldig, G., Green-Petersen, D. M. B., Nielsen, N. S., Baron, C. P., Jacobsen, C. (2013). Oxidative stability and sensory attributes of fermented milk products fortified with a neat or pre-emulsified mixture of fish oil and marine phospholipids. *Journal of the American Oil Chemists' Society (resubmitted after revision)*.

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- VII. Lu, F. S. H., Nielsen, N. S., & Jacobsen, C. (2012). Short Communication: Comparison of two methods for extraction of volatiles from marine PL emulsions. *European Journal of Lipid Science and Technology*, 115, 246-251.

TABLE OF CONTENT

PREFACE	i
ACKNOWLEDGEMENTS	ii
SUMMARY	iii
SAMMENFATNING	v
LIST OF PUBLICATIONS	ix
CHAPTER 1 INTRODUCTION	1
1.1 Objectives:	2
CHAPTER 2 MARINE PHOSPHOLIPIDS	4
2.1 Classification and sources of marine phospholipids	4
2.2 Antioxidative effect of marine PL	6
2.3 Food fortification with n-3 fatty acids from marine lipids.....	9
CHAPTER 3 LIPID OXIDATION AND MARINE PL EMULSIONS	11
3.1 Autoxidation of marine PL	11
3.2 Formation of secondary volatiles derived from marine PL.....	15
3.2.1 Secondary volatiles derived from n-3 LC PUFA	15
3.2.2 Secondary volatiles derived from non-enzymatic browning reactions	17
3.3 Physico-chemical of marine PL emulsions and liposomal dispersions.....	18
3.4 Factors that influence lipid oxidation in emulsions	20
3.4.1 Effect of antioxidant toward lipid oxidation in marine PL emulsions	23
3.4.2 Effect prooxidants toward lipid oxidation in marine PL emulsions.....	24
CHAPTER 4 NON-ENZYMATIC BROWNING IN MARINE PL	26
4.1 Non-enzymatic browning produced as a consequence of lipid oxidation.....	27
4.2.1 Strecker degradation.....	28
4.2.2 Pyrroles formation and polymerization	32
4.2.3 Antioxidative properties of pyrroles.....	33
4.2.4 Antioxidative activity of pyrroles in oxidized PL.....	35
4.2.5 Effect of tocopherol on the antioxidative activity of pyrroles	37

4.3 Non-enzymatic browning in marine PL liposomes.....	38
CHAPTER 5 EXPERIMENTAL WORK	39
5.1 An overview of marine PL preparations used in the present Ph.D. study.....	39
5.2 Experimental approach	42
5.2.1 Part 1: Evaluation of physico-chemical properties of marine PL emulsions (paper II).....	42
5.2.2 Part 2: Evaluation of oxidative stability in marine PL emulsions (paper III & IV)	44
5.2.3 Part 3: Evaluation of non-enzymatic browning reactions in marine PL (paper III & V)....	48
5.2.4 Part 4: Evaluation of marine PL fortified foods (paper VI).	49
5.3 Methodology	52
5.3.1 Characterisation of marine PL (paper II-IV).....	52
5.3.2 Physico-chemical properties of marine PL emulsions (paper II)	52
5.3.3 Hydrolytic and oxidative stability of marine PL (paper II-V).....	52
5.3.4 Non-enzymatic browning reactions in marine PL (paper III-V).....	52
5.3.5 Sensory evaluation (paper VI)	53
5.3.6 Statistical analysis (paper II - VI).....	53
CHAPTER 6 SUMMARY OF RESULTS AND DISCUSSION	54
6.1 Part 1: Physico-chemical properties of marine PL emulsions (paper II)	54
6.1.1 A summary of physico-chemical properties of marine PL emulsions	54
6.1.2 Discussion of physical stability of marine PL emulsions.....	56
6.2 Part 2: Oxidative stability of marine PL emulsions (paper III & IV)	57
6.2.1 A summary of oxidative stability of marine PL emulsions/dispersions.....	58
6.2.2 Discussion of oxidative stability of marine PL emulsions/dispersions	59
6.3 Part 3: Non-enzymatic browning reactions in marine PL (paper III & V)	61
6.3.1 A summary of non-enzymatic browning reactions in untreated marine PL emulsions.....	62
6.3.2 A summary of non-enzymatic browning reactions in purified marine PL dispersions.	63
6.3.3 Proposed mechanisms for non-enzymatic browning reactions in marine PL.....	63
6.3.4 Discussion of lipid oxidation and non-enzymatic browning in marine PL.....	65
6.4 Part 4: Food fortification with marine PL (paper VI)	68

6.4.1 A summary of findings for marine PL fortified food (fermented milk products).....	68
6.4.2 Discussion of findings and the potential use of marine PL for food fortification.....	70
CHAPTER 7 CONCLUSION AND FUTURE PERSPECTIVES.....	73
LIST OF REFERENCES.....	76
APPENDIX.....	88

CHAPTER 1

INTRODUCTION

Marine phospholipids (PL) have been the focus of much attention recently. Many studies have shown that marine PL provide more advantages than marine triglycerides (TAG) available from fish oil. These advantages include: i) a higher content of physiologically important n-3 long chain polyunsaturated fatty acids (LC PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Peng et al., 2003); ii) a better bioavailability of EPA and DHA (Wijendran et al., 2002); iii) a broader spectrum of health benefits including those from n-3 LC PUFA, their polar head groups and the combination of the two in the same molecule (Ierna et al., 2010); iv) a better resistance towards oxidation due to the antioxidative properties of PL (Cho et al., 2001; Moriya et al., 2007).

The issues on health benefits and oxidative stability of marine PL have been discussed and summarized in paper I and therefore will not be further discussed here. The oxidative stability of marine PL is summarized as follows: A high oxidative stability of marine PL might be due to a) their tight intermolecular packing conformation at the sn-2 position (Applegate & Glomset 1986) and b) synergistic effect of phospholipids on antioxidant activity of α -tocopherol, which is also present in marine PL (Moriya et al., 2007). In addition, recent studies (Hidalgo et al., 2005) showed that pyrroles, the antioxidative compounds resulting from non-enzymatic browning between oxidized amino phospholipids/amino acids and fatty acid oxidation products in slightly oxidized marine PL also had protective effect against oxidation. Among these factors, synergistic effect of phospholipids on antioxidant activity of α -tocopherol seems to be the main reason for the extraordinary stability of marine PL as suggested by several studies (Cho et al., 2001; Moriya et al., 2007).

Due to the numerous advantages of marine PL, there is a growing awareness about the potential use of marine PL as ingredient for food fortification. Marine PL have a high content of phosphatidylcholine, which has amphiphilic properties. Therefore, marine PL are potential natural surfactants for emulsion preparation. Furthermore, marine PL emulsions can be used as effective carriers of n-3 LC PUFA rich oil as they can be incorporated easily into aqueous and emulsified foods. To date, many studies on n-3 TAG fortified functional foods are available in literature; food fortification with marine PL has scarcely been studied. There are only a few studies regarding the oxidative stability of marine PL liposomes or marine PL based liposomes under gastrointestinal condition (Cansell et al., 2001; Nacka et al., 2001a; 2001b; Mozuraityte et al., 2006a; 2006b; 2008). The limited applications of marine PL in

food industry could be attributed to several reasons such as the lack of knowledge, especially relating to behaviour of marine PL in food systems, the quality of marine PL that are available in the market and limitations in large scale production of liposomes without using organic solvent for food applications. Due to the high content of n-3 LC PUFA in marine PL, foods fortified with marine PL are still susceptible to oxidation despite the high oxidative stability of marine PL. Oxidation of marine PL might result in oxidative products that not only could cause deterioration of food quality and the generation of off-favours but also could increase the risk of certain degenerative diseases.

In addition, marine PL have more complex composition and lower purity than TAG fish oil as they are not refined and deodorized as fish oils are. Several recent studies (Hidalgo et al., 2003; 2005a; 2005b; 2006; 2007) have reported the occurrence of non-enzymatic browning reactions in a model system or matrix containing phosphatidylethanolamine (PE) and amino acids. Thus, it is speculated that non-enzymatic browning reactions might occur in marine PL particularly if they contain primary amine groups from PE or amino acid residues. The interaction between non-enzymatic browning reactions and lipid oxidation may complicate the study of oxidative stability of marine PL. Therefore, more comprehensive studies are required to investigate the oxidative stability and sensory properties of marine PL prior to exploring their potential uses in food industry.

1.1 Objectives:

The main objective of this Ph.D. research was to explore the possibilities of using marine PL for food fortification. In order to achieve this main objective, this Ph.D. research was divided into 4 more specific objectives in different parts: Part 1) to investigate the physico-chemical properties of marine PL emulsions, Part 2) to investigate the hydrolytic and oxidative stability of marine PL emulsions, Part 3) to investigate the non-enzymatic browning reactions in marine PL emulsions, Part 4) to investigate the sensory properties and oxidative stability of selected foods fortified with marine PL. Overall, this Ph.D. research also identified the important factors affecting the stability of marine PL in both emulsions and real food systems. The hypotheses behind these parts are described as follows:

Hypotheses in part 1:

- a) It is possible to use marine PL to prepare emulsions as a n-3 LC PUFA delivery system without addition of other emulsifiers. Likewise, it is possible to use marine PL to prepare physically stable fish oil emulsions with a sufficient amount of marine PL as emulsifier.
- b) The physical stability of marine PL emulsions varies depending on the ratio of fish oil and marine PL, as well as the type of PL used as surfactant (chemical composition of marine PL) for emulsion preparation.

Hypotheses in part 2:

- a) Emulsions prepared from marine PL containing n-3 LC PUFA in PL form are more oxidatively stable as compared to emulsions prepared from fish oil containing n-3 LC PUFA in TAG form.
- b) The oxidative stability of marine PL emulsions varies depending on the quality, source and chemical composition of marine PL used.
- c) α -tocopherol is an efficient antioxidant to maintain the high oxidative stability of marine PL.

Hypotheses in part 3:

- a) Non-enzymatic browning reactions occur in marine PL emulsions due to the interaction between lipid oxidation products with the primary amine group from PE or residues of amino acids/protein that are present in marine PL.
- b) Non-enzymatic browning reactions might affect lipid oxidation in marine PL emulsions or vice versa.

Hypotheses in part 4:

- a) It is possible to incorporate marine PL either in neat or pre-emulsified form into real food systems without adversely affecting the oxidative stability and sensory quality of fortified foods.
- b) The oxidative stability and sensory quality of marine PL fortified foods vary depending on the quality and source of marine PL used for fortification.

2.1 Classification and sources of marine phospholipids

Phospholipids can be categorized into three major classes: glycerophospholipids, ether glycerolipids and sphingophospholipids. Among them, glycerophospholipid is the most widespread class and comprises phospholipids with different polar head groups. For example, phosphatidylcholine has choline as a head group, while phosphatidylethanolamine has ethanolamine as a head group, etc., as shown in Figure 2.1. Therefore, the discussion of phospholipids in the present Ph.D. thesis is mainly focus on glycerophospholipids with an abbreviation of PL and abbreviations for phospholipids in this category are listed as follows: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SPM) and lysophosphatidylcholine (LPC).

The chain length and the degree of unsaturation of two fatty acids located at sn-1 and sn-2 positions of PL may vary from source to source. For instance, marine PL are rich in EPA and DHA, which have chain lengths of 20 and 22 carbon atoms with 5 and 6 double bonds, respectively. Moreover, most of the PL originating from marine sources such as PC has a polyunsaturated fatty acid (PUFA) at sn-2 position, while PL originating from plants such as soybean PC does not have a PUFA at sn-2 position (as shown in Figure 2.2). Thus, the most dominant molecular species are C16:0-20:5 PC or C16:0-22:6 PC and C18:2-18:2 PC or C16:0-18:2 PC in marine PC and soybean PC, respectively (Le Grandois et al., 2009). As far as marine sources are concerned, PL were found relatively abundant in roe, fish heads and offals such as viscera (Falch et al., 2006). As shown in Table 2.1, the most abundant PL in marine sources such as salmon, tuna, rainbow trout and blue mackerel is PC, followed by PE and other minor PL such as PI, PS, SPM and LPC. Furthermore, krill such as *Euphausia Superba* and *Euphausia Pacifica* are other rich sources of marine PL (Saito et al., 2002; Le Grandois et al., 2009). For instance, Neptune Krill oil (a concentrate of marine PL from *Euphausia Superba*) is a leading commercial krill oil in the current market.

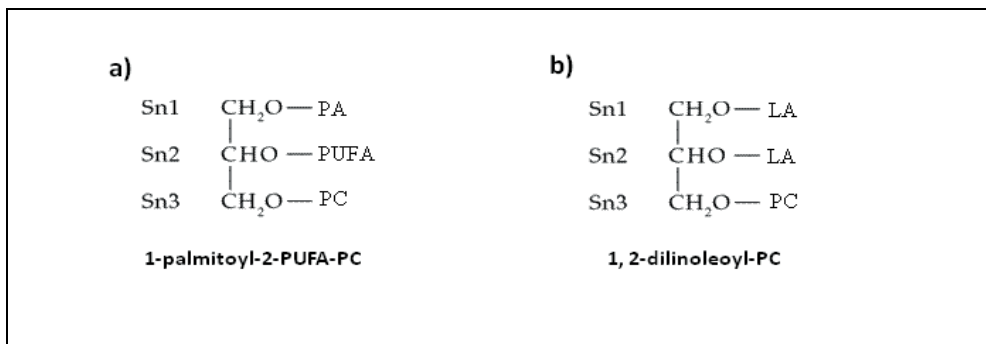


Figure 2.2 Most dominant molecular species in a) marine PL and b) soybean PL (Miyashita et al., 1994).

2.2 Antioxidative effect of marine PL

The issue on oxidative stability of marine PL has been discussed extensively in a Paper I and thus only a brief summary of this topic is given here. Several studies have shown that marine PL have a high oxidative stability (King et al., 1992a; 1992b; Boyd et al., 1998; Belhaj et al., 2010). A number of hypotheses have been suggested to explain the high oxidative stability in marine PL: *a) conformation of PUFA at the sn-2 position* (Applegate & Glomset 1986; Miyashita et al., 1994; Nara et al., 1997). A study of Miyashita and co-workers (1994) showed that salmon roe PC had higher oxidative stability than soybean PC in an aqueous solution dispersed with chicken egg albumin despite the higher degree of unsaturation in the salmon roe PC. This was suggested to be due to the presence of the main molecular species in salmon roe PC, 1-palmitoyl-2-PUFA-phosphatidylcholine (with most of the PUFA located at sn-2 position of PC), which provide a more tightly packed molecular conformation as compared to that of soybean PC (1, 2-dilinoleoyl-phosphatidylcholine). Consequently, it is difficult for free radicals and oxygen to attack PUFA in bilayers of tighter conformation in salmon roe PC liposomes. Further details of this tighter molecular confirmation can be found in paper I. The same observation was obtained by Nara and co-workers (1997; 1998), who reported that aqueous micelles or liposomes prepared from salmon roe PC have a better oxidative stability than chicken egg PC and soybean PC.

Table 2.1 Phospholipid composition (%) of marine sources^a

PL classes	Salmon head lipids	Rainbow trout fillet lipids	Bigeye muscle lipids	Bluefin muscle lipids	Bonito muscle lipids	Frigate muscle lipids	Skipjack muscle lipids	Yellowfin muscle lipids	Krill	Salmon roe
PC	54.7	53.6	42.1	42.2	53.9	47.4	51.5	37.9	87.5	86.0
PE	14.0	22.9	18.8	18.9	20.1	21.8	20.2	21.0	6.3	6.0
PI	2.5	8.3	5.8	6.7	2.3	10.9	4.9	8.5	0.5	2.0
PS	10.4	4.1	5.4	4.8	2.2	5.1	5.0	5.4	0.5	ND
SPM	8.3	4.9	3.3	5.6	7.6	3.0	0.5	4.0	1.3	2.0
LPC	1.4	ND	22.1	15.4	13.8	12.0	18.3	21.5	ND	2.0
Cardiolipin	ND	6.2	ND	ND	ND	ND	ND	ND	ND	ND
Other	ND	ND	4.4	6.6	Trace	1.7	1.5	2.8	3.9	1.0

^a Data compiled from several studies (Neptune Technologies & Bioresources, 2001; Gbogouri et al., 2006; Striby et al., 1999; Medina et al., 1995; Body & Vlieg, 1989; Schneider, 2008). PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SPM, sphingomyelin and LPC, lysophosphatidylcholine, ND = not determined.

b) synergistic effect of phospholipids on the antioxidant activity of α -tocopherol, which is also present in marine PL (Cho et al., 2001; Moriya et al., 2007). Cho and co-workers (2001) reported that a better oxidative stability was found in the lipid fractions from three kinds of squid tissue (viscera, muscle and eye), total lipids (TL) and trout egg TL as compared to that of bonito TAG and tuna orbital TL. This was suggested to be due to the presence of PL in the lipid fractions from squid tissue and trout egg. In addition, Moriya and co-workers (2007) reported that lipid fractions from fish roe (salmon roe and herring roe) were more oxidatively stable than commercial fish oils (crude tuna oil and crude sardine oil) despite the higher level of PUFA and lower level of tocopherol in fish roe. They proposed that the high content of PL or the synergistic effect of PL on antioxidant activity of α -tocopherol in fish roe was the main reason for its better oxidative stability. Furthermore, several studies (Kashima et al., 1991; Weng & Gordon, 1993; Bandara et al., 1999) also reported that the synergistic effect of PE with α -tocopherol was higher than that of PC. For instance, Bandara and co-workers (1999) investigated the prevention of lipid oxidation in a refined sardine oil system with added α -tocopherol at 0.04 %, or with added PC, PE and cardiolipin (CL) at 0.5 %, respectively. They reported that PC was the most effective individual antioxidant when it was compared to PE, CL and α -tocopherol while the highest synergistic effect was provided by PE. This could be due to the ease hydrogen transfer from the amine group of PE to tocopheroxyl radical and regeneration of tocopherol or the secondary antioxidant action of PE in reducing quinones formed during oxidation of tocopherols (Weng & Gordon, 1993).

In addition, c) recent studies (Hidalgo et al., 2005b) showed that *pyrroles, antioxidative compounds resulting from non-enzymatic browning* (reactions between oxidized PE/amino acids and the fatty acid oxidation products in slightly oxidized marine PL) also have antioxidative properties. Antioxidative effect of pyrroles will be further discussed in chapter 4. Among all the factors mentioned above, several studies (Cho et al., 2001; Moriya et al., 2007) suggested that the synergistic effect of PL on the antioxidant activity of α -tocopherol seems to be the main reason for the extraordinary stability in marine PL.

2.3 Food fortification with n-3 fatty acids from marine lipids

As mentioned earlier, marine lipids have numerous health benefits, especially the strong and consistent cardio-protective effect demonstrated by EPA and DHA. Unfortunately, EPA and DHA cannot be synthesized endogenously in human body. There is only a low conversion rate of α -linolenic acids (ALA) to EPA and DHA in human body as shown by several studies (Hussein et al., 2005; Pawlosky et al., 2001). All these reasons have prompted a number of organisations to recommend higher intakes of these n-3 fatty acids. Examples on guidelines of n-3 fatty acids intake are stated as follows: a) the British Nutrition Foundation has recommended a daily intake of 1.25 g EPA/DHA for normal adult (British Nutrition Foundations's Task Force, 1992), b) the International Society for the Study of Fatty Acids and Lipids (ISSFAL, 2004) has recommended an adequate intake of EPA and DHA to be 500 mg, c) the American Heart Association (2002) has recommended fish intake, particularly fatty fish at least 2 times per week, d) European Food Safety Authority (EFSA, 2010) has recommended a daily intake of 250 mg/day long chain n-3 PUFA for adults to reduce the risk of heart disease. In addition, the daily intake of n-3 fatty acids must not exceed 2 g per day. Currently, there is no guideline for a recommended dosage for marine PL supplement such as krill oils intake.

Despite the beneficial effects of n-3 fatty acids, the fish consumption is generally still low in many societies as fresh fish is not always available and some people do not like to eat fish. Thus, fish oil/krill phospholipids supplement or food fortification with n-3 fatty acids in the form of TAG/PL is a dietary alternative to improve the low fish consumption. However, the most natural way to increase the intake of n-3 fatty acids is through food fortification, especially the foods that are regularly consumed by a majority of population. Currently, there is a wide range of n-3 fatty acids in the form of TAG oil and powder that are available for food fortification in the market (Trautwein, 2001). As far as the TAG n-3 fatty acids fortified foods are concerned, the infant formulas and baby follow-on foods were the products that spearheaded the n-3 fatty acids fortified foods in the market. Gradually, products such as margarines, low fat spread, bread, UHT and full fat milk, yoghurt, fruit juices and beverages also entered the mainstream, followed by niche products such as salad dressings, soups, iced-tea drink, biscuits, cakes and n-3 fatty acids fortified canned seafoods (Whelan & Rust, 2006; Kolanowski & Laufenberg, 2004; 2006).

The use of marine PL for food applications is a new area in food industries. There is no current use of marine PL for food application has been reported. However, several krill oil companies have taken attempts toward this direction. For instance, Enzymotec has obtained a Generally Recognized as Safe (GRAS) status for their krill derived lecithin for the use in breakfast bars, soy products, fat spreads, milk based beverages, yoghurt and soft candy in the range of 0.6 % to 3.8 % (FDA 2008a). In addition, both Aker Biomarine, and Neptune Technologies and Bioessource also have obtained a GRAS status for their Superba™ krill oil and Neptune krill oil, respectively for the use as a food ingredient in non-alcoholic beverages, breakfast cereals, cheeses, frozen dairy desserts, whole and skin milk, processed fruit and fruit juices, and medical foods at levels ranging from 0.05 to 0.50 g per serving (FDA 2008b; 2011).

There are numerous studies on n-3 fatty acids fortified foods available in literature, particularly focus on fortification with TAG fish oil. For instance, studies on fish oil fortified ice-cream (Rudolph, 2001), mayonnaise (Jacobsen et al., 2003), spread (Dalton et al., 2006), milk (Let et al., 2007), drinking yoghurt (Nielsen et al., 2007), spaghetti (Verardo et al., 2009), bread (Lu & Norziah, 2010; 2011), etc. To the best of my knowledge, only few studies on marine PL food fortification, namely krill oil fortification are available in literature. For instance, fortification of surimi seafood with n-3 fatty acids rich oils from flaxseed, algae, menhaden, krill and a blend of these oils (Pietrowski et al., 2011). They reported that fortification of surimi seafood with krill oil not only increased the n-3 fatty acids content of the product but also increased the susceptibility of this product towards lipid oxidation. This phenomenon was due to the high content of EPA and DHA in krill oil, but the lipid oxidation of the fortified product was still within ranges acceptable to consumers. In addition, the above research group also studied the sensory properties, lipid composition and antioxidant capacity of novel nutraceutical egg products developed with same n-3 fatty acids rich oils as mentioned earlier (Kassis et al., 2011; Sedoski et al., 2012). Their results showed that all fortified egg products with n-3 fatty acids rich oils including krill oils were acceptable to consumers and had potential market in future.

CHAPTER 3 LIPID OXIDATION AND MARINE PL EMULSIONS

Phospholipids (PL) are degraded through main pathways of hydrolysis and/or oxidation. Hydrolysis usually occurs in the presence of water to produce lysophospholipids and free fatty acids. Lysophospholipids are subsequently degraded to glycerophospho compounds as the end product of PL hydrolysis. However, the hydrolysis of PL emulsion is minimal at neutral pH as PL hydrolysis is catalyzed by hydroxyl and hydrogen ions (Gritt et al., 1993). On the other hand, the PL degradation via oxidation of its fatty acids is similar to other lipids. Marine PL are susceptible to oxidation in the presence of catalysts/initiators such as transition metals (iron and copper), light, heat, enzymes (lipoxygenases), metalloproteins, and microorganisms leading to lipid autoxidation, photooxidation, thermal, and enzymatic or non-enzymatic oxidation. In the present Ph.D. study, emulsions were prepared from a combination of marine PL with fish oil and the storage of emulsions were carried out in darkness at low temperature, thus the discussion of photooxidation, thermal and enzymatic oxidation is not the main focus of this study. The discussion of this section will mainly focus on mechanisms of autoxidation with special emphasis on n-3 LC PUFA, namely EPA and DHA.

3.1 Autoxidation of marine PL

Similar to the oxidation of TAG in fish oil, the n-3 LC PUFA chains in marine PL are the primary targets of oxidation. Autoxidation of n-3 LC PUFA in PL occurs via a free radical chain reaction that can be divided into 3 stages: initiation, propagation and termination. A simplified scheme of lipid autoxidation is given in Figure 3.1.

Initiation:

Unsaturated lipid molecules or fatty acids lose a hydrogen atom and generate free radicals in the presence of initiators. The abstraction of hydrogen radical ($H\cdot$) normally occurs at the bis-allylic positions of polyunsaturated fatty acids (PUFA), which is the rate-limiting step in lipid oxidation. Therefore, the susceptibility of PUFA to oxidation depends on the availability of bis-allylic hydrogens. Oxidative stability of PUFA is inversely proportional to the number of bis-allylic positions in the molecule or the degree of unsaturation of the PUFA. For instance, EPA and DHA have four and five active bis-allylic methylene groups, respectively. Thus, the

reactivity of DHA is approximately 5 times greater than that of linoleic acid (Kulas et al., 2003) because the rate of autoxidation of PUFA increases approximately 2 times for each active bis-allylic methylene group (Frankel 2005). However, the oxidative stability of PUFA might be in reverse order in multiphase or liposome system (Miyashita et al., 1993). As mentioned earlier in Chapter 2, this phenomenon is due to the conformation of the fatty acids in the micelles (e.g. the unsaturated part of the fatty acids buried in the hydrophobic interior of the micelles).

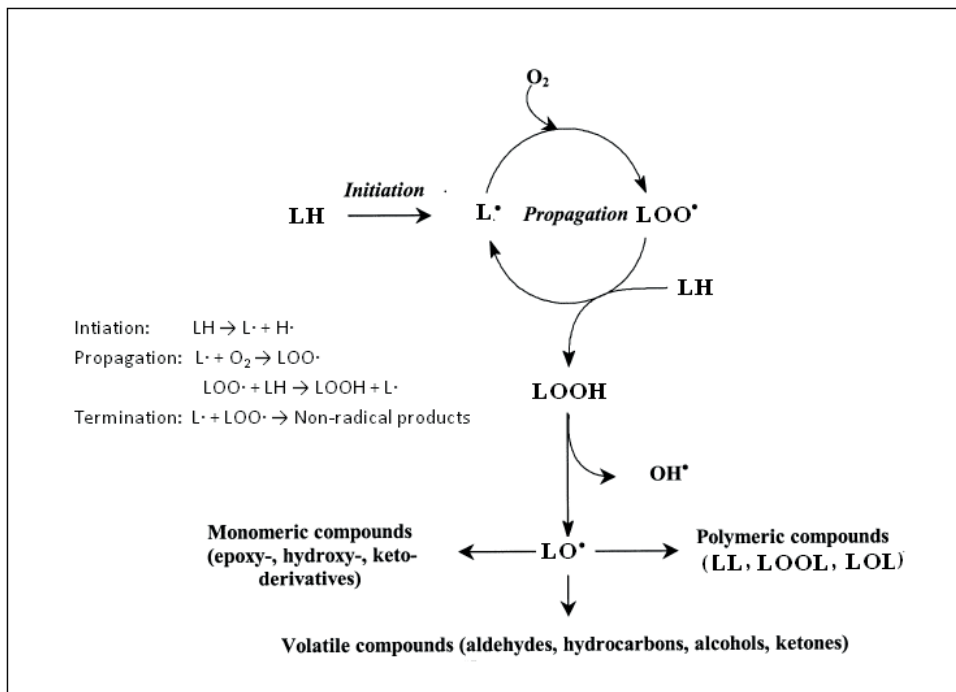


Figure 3.1 Oxidation mechanisms of polyunsaturated lipids. LH[•]: Unsaturated lipid; X[•]: Radical initiator; L[•]: Lipid alkyl radical; LO[•]: Lipid alkoxy radical; LOO[•]: Lipid peroxy radical; LOOH: Lipid hydroperoxide (Adapted from Frankel, 2005; Dobarganes & Marquez-Ruiz, 2007).

Propagation:

The alkyl radical (L[•]) produced from the initiation stage reacts quickly with triplet oxygen to generate peroxy radicals (LOO[•]). Peroxy radicals are not stable and they abstract hydrogen atoms from another unsaturated lipid molecule to form hydroperoxides and another alkyl radical. This reaction is repeated thousands of times during the propagation stage until no hydrogen source is available or the chain is interrupted by antioxidants. For instance,

oxidation of EPA and DHA can produce mixtures of eight and ten positional hydroperoxides isomers, respectively; with 5-, 8-, 9-, 11-, 12-, 14-, 15- and 18 hydroperoxides derived from EPA, whereas 4-, 7-, 8-, 10-, 11-, 13-, 14-, 16-, 17-, and 20- hydroperoxides derived from DHA. Decomposition of hydroperoxides derived from EPA and DHA to produce secondary volatiles will be further discussed in section 3.2. Meanwhile, alkoxy (LO^\bullet), peroxy (LOO^\bullet), hydroxyl ($^\bullet\text{OH}$) and new lipid radical (L^\bullet) generated from the decomposition of hydroperoxides further participate in the chain reaction of free radicals.

Termination:

Lipid oxidation is terminated when lipid radicals react together to form stable non-radical products which do not further participate in the radical chain reaction. In addition, termination also occurs when lipid radicals react with antioxidants (Frankel, 2005). Mechanisms of antioxidant in preventing lipid oxidation are described in section 3.4.1.

Autoxidation of lipids produce a great variety of compounds with different polarities, stabilities and molecular weights. These compounds can be classified as three main groups as suggested by Dobarganes & Marquez-Ruiz (2007): a) compounds with molecular weights similar to those of the unsaturated lipid molecules (LH) but with one of their fatty acids undergone oxidation, b) volatile compounds such as aldehydes, hydrocarbons, alcohols and ketones (this part will be further discussed in section 3.2), c) polymerization compounds such as dimers or polymers, which are formed through the interactions of two lipid radicals (L^\bullet) and therefore they have higher molecular weights than those LH.

Dimers and polymers are large molecules that are formed by a combination of $-\text{C}-\text{C}-$, $-\text{C}-\text{O}-\text{C}-$ and $-\text{C}-\text{O}-\text{O}-\text{C}-$ bonds (Kim et al., 1999). They have either acyclic or cyclic structures depending on the reaction process and types of fatty acids in lipids (Tompkins & Perkins, 2000). Polymerisation usually occurs at the accelerated stage of oxidation or at high temperature when the solubility of oxygen decreases drastically and most of the hydroperoxides (LOOH) are decomposed to form peroxy (LOO^\bullet) and alkoxy radicals (LO^\bullet). In such condition, the most dominant reaction is initiation stage of lipid oxidation and the concentration of alkyl radicals (L^\bullet) is higher than alkyl peroxy radicals (LOO^\bullet). Therefore, oxypolymers are formed through reaction mainly involving alkyl radicals (L^\bullet) and alkoxy radicals (LO^\bullet). According to Khayat & Schwall (1983), oxypolymerisation of lipid

3.2 Formation of secondary volatiles derived from marine PL.

Under certain conditions such as high temperature and presence of transition metal ions, unstable lipid hydroperoxides may decompose through the formation of peroxy and alkoxy radicals, and cleavage of the alkoxy radicals by homolytic β -scission to form a wide variety of shorter-chain secondary oxidation volatiles. Marine PL have a more complex matrix than fish oil as marine PL may contain amino acids residues or protein in addition to the high n-3 PUFA content in glycerophospholipids. Thus, it is speculated that marine PL have a broader spectrum of secondary volatiles, including those derived from n-3 LC PUFA and those from non-enzymatic reactions, reactions between lipid oxidation products with the primary amine groups from PE or amino acids/proteins that are present in marine PL.

3.2.1 Secondary volatiles derived from n-3 LC PUFA

To the best of my knowledge, study on the characterizations of marine PL-derived volatiles is scarcely available in literature. Several studies have investigated the secondary volatiles derived from n-3 LC PUFA in bulk fish oil system (Karahadian et al., 1989; Aidos et al., 2002) and real food systems such as milk, mayonnaise, etc (Hartvigsen et al., 2000; Venkateshwarlu et al., 2004; Sørensen et al., 2010a; 2010b). Although the primary oxidation products of n-3 LC PUFA themselves are tasteless and odourless, decomposition of these products such as ketones and aldehydes that have low odour thresholds may adversely affect the flavour, taste and overall quality of foods containing n-3 PUFA. For instance, volatiles such as 1-penten-3-one, (*Z*)-4-heptenal, 1-octen-3-one, 1, 5-octadien-3-one, (*E, E*)-2, 4-heptadienal, and (*E, Z*)-2, 6-nonadienal derived from n-3 LC PUFA have been reported as the most potent odorants in fish oil. Despite the potency of these volatiles, none of this individual volatile but rather a combination of volatiles is responsible for a fishy or metallic off-flavour in fish oil enriched milk (Venkateshwarlu et al., 2004). Some of the selected n-3 LC PUFA derived volatiles and their associated odours are listed in Table 3.1. In fact, the selected volatiles also present in marine PL emulsions prepared in this study.

Table 3.1 Some of the selected n-3 LC PUFA derived secondary volatiles and their odours.

Volatiles	Odour description	References
Propanal	Sharp, irritating, plastic	<i>c</i>
(<i>Z</i>)-4-Heptenal	Creamy, stale, burnt, fishy	<i>a, b</i>
(<i>E, Z</i>)-2, 4-Heptadienal	burnt, fishy, fatty	<i>a, b, c</i>
(<i>E, E</i>)-2, 4-Heptadienal	Fishy, rancid, green	<i>a, b, c</i>
(<i>E, Z</i>)-2, 6-Nonadienal	fresh cucumber, green, melon	<i>a, b, c</i>
(<i>E, E</i>)- 2, 6-Nonadienal	deep fried, fatty, cucumber,	<i>b,</i>
1-penten-3-one	pungent, fishy, plastic	<i>b, c</i>
(<i>E</i>)-2-Hexenal	green	<i>c</i>
1-octen-3-one	mushroom	<i>b, c</i>
1, 5-octadien-3-one	metallic	<i>a, b, c</i>

The information is adapted from references: a) Karahadian et al. (1989); b) Hartvigsen et al. (2000); c) Venkateshwarlu et al. (2004).

3.2.2 Secondary volatiles derived from non-enzymatic browning reactions

In the present Ph.D. study, volatiles derived from non-enzymatic browning reactions have been identified in emulsions prepared from marine PL or purified marine PL with amino acids added. Thus, selected volatiles derived from non-enzymatic browning reactions as reported by several studies were chosen for comparison as follows (Table 3.2):

Table 3.2 Some of the selected volatiles from seafood products and model systems containing primary amine group.

Volatiles	previously reported in products/ derived from amino acids	reported in papers
dimethylsulphide ^{a, d}	shrimp, anchovy, oyster/ methonine ^d	
dimethyl disulphide ^{a, b, d}	scallop, oyster / methonine ^d	III, V
dimethyl trisulphide ^{c, d}	crab / methonine ^d	III
pyridines ^{a, b, c}	scallop, crab	III
3-methylpyridine ^{a, b}	scallop	
trimethylpyrazine ^c	crab	III
3-ethyl-2, 5-diethylpyrazine		III,
2, 3-dimethylpyrazine ^{a, b}	shrimp (raw, fermented, cooked),	
2, 5-dimethylpyrazine ^b	roasted squid, clam	
2-methyl-2-pentenal ^{a, b, e}	oyster, anchovy, scallop/ lysine ^e	III, V
3-methylbutanal ^c	crab / leucine ^d	III, IV, V
2-methylbutanal ^c	crab / leucine ^d	III,
benzaldehyde ^{a, d}	cooked crayfish, oyster, shrimp	III,
2-methyl-2-butenal ^{b, e}	dried scallops / lysine ^e	III, V
2-pentylfuran ^{b, d}	dried scallops	III
2-methylpropanal ^a	roasted dried squid, anchovy	III

The information of this table is adapted from references: a) fresh adductor muscle and total lipids of sea scallop (Linder & Ackman, 2002); b) dried scallops (Chung et al., 2001); c) steamed mangrove crab (Yu & Chen, 2010); d) model system containing liposomes prepared from Longissimus dorsi muscle and selected amino acids, namely phenylalanine, methionine and leucine (Ventanas et al., 2007), e) model system containing (E)-4,5-epoxy-(E)-2-heptenal and lysine or bovine serum albumin (Zamora & Hidalgo, 1994).

Several studies (Flores et al., 1998; Ventanas et al., 2007) suggested that 2-methylbutanal and 3-methylbutanal are Strecker degradation products from isoleucine or leucine, respectively while dimethylsulphide, dimethyldisulphide and dimethyl trisulphide are degraded from methionine. In addition, 2-methyl-2-pentenal and 2-methyl-2-butenal were suggested to be the major volatiles resulting from a reaction between (*E*)-2-(*E*)-4-heptadienal with a lysine (Zamora & Hidalgo., 1994). Pyrazines and pyridines are thermal products generated via Strecker degradation from various nitrogen sources in heat processed foods (Whitfield, 1992; Wong & Bernhard, 1998; Chung et al., 1999).

3. 3 Physico-chemical of marine PL emulsions and liposomal dispersions

An emulsion system normally comprises three regions: a) interior of droplet, b) continuous phase and interfacial region. The interfacial region is a region surrounding each emulsion droplet and comprises a mixture of oil, water and emulsifier molecules. Basically, emulsion can be distinguished by the composition of the dispersed and continuous phases. There are two types of emulsions: a) oil-in-water (o/w) emulsion, a system consisting of oil droplets dispersed in aqueous phase; b) water-in-oil (w/o) emulsion, a system consisting of water droplets dispersed in an oil phase. In the present Ph.D. study, oil-in-water emulsion was used as a n-3 LC PUFA delivery system for food fortification and therefore discussion is mainly focused on o/w emulsion.

It is widely accepted that emulsion is a thermodynamically unstable system and it tends to break down over time. Three of these major breakdown processes include flocculation, creaming and coalescence. Flocculation occurs when two or more droplets that keeps their integrity aggregate. Flocculation is often the first stage of emulsion destabilization, followed by creaming and coalescence. Creaming occurs due to the differences in density between oil and aqueous phase. For instance, oil droplets of lower density than water phase move upward and accumulate at the top solution in a creamed layer. Coalescence occurs in emulsions especially in the absence of an emulsifier when droplets collide and merge into larger droplets. Thus, emulsifiers/surfactants are used to cover the oil droplets and reduce the interfacial tension for emulsions stabilization (McClements & Decker, 2000). In general, the stabilization of droplets in o/w emulsions can be achieved through two main mechanisms: a) electrostatic stabilization, which arises from the electrostatic repulsion between droplets in emulsions. The electrostatic repulsion occurs due

to absorption of charged surfactants at the oil-water interface. The magnitude and sign of the electrical charge of droplets depend on the type and concentration of charged surface-active surfactants and the pH of the emulsion. An example of electrostatic stabilization is given by an o/w emulsion prepared from a mixture of TAG and PC, which has zeta potential ranges +10 to +60mV, demonstrating the electrostatic repulsion of PL (Arts et al., 1994). b) steric stabilization, which results from the absorption of macromolecules such as polysaccharides or soluble protein to the droplet interface.

Marine PL contain a high level of phosphatidylcholine (PC) which has amphiphilic properties and thereby marine PL are potential surfactants for emulsion preparation (Bueschelberger, 2004). In addition, PC from marine PL can self-assemble to form a variety of thermodynamically stable structures including micelles and bilayer vesicles/liposomes (Coupland & McClements, 1996). Several studies have investigated the dispersal mechanism of vegetable oil in soybean PC to form o/w emulsions (Asai & Watanabe, 1999; Asai, 2003). They reported that the coexistence of PC monolayer encased oil droplets and PC liposomes are crucial to stabilize this kind of o/w emulsions as PC bilayers have maximum value of spreading pressure. In addition, these studies reported that a stable dispersion could be obtained when PC mole fraction was more than 0.03 (or oil fraction less than 0.95). This is because a sufficient amount of PC monolayer was required to cover the oil droplets completely and to avoid drastic increase of droplet sizes and the separation of emulsions into oil and water. They recommended oil fractions of 0 to 0.8 in order to obtain a stable PC o/w emulsion.

Furthermore, stabilisation of o/w emulsion is greatly influenced by the molecular geometry of a surfactant molecule/emulsifier. This molecular geometry can be described by a packing parameter, p (Israelachvili, 1992, 1994):

$$p = v/L.a \quad \text{Equation 3.1}$$

where v and L are the molecular volume and length of the hydrophobic tail and a is the cross-sectional area of the hydrophilic head group.

When surfactant molecules associate with each other in the formation of small oil droplets and the stabilisation of o/w emulsion, they tend to form monolayers that have an optimum curvature. This optimum curvature allows monolayer to have its lowest free energy and most efficient packing of the molecules. The optimum curvature (H_0) of a monolayer depends on the packing parameter (p) of the surfactant (as shown in Figure 3.3). For instance, PC comprising two lipophilic fatty acids and a large polar head group exhibits $p = 1$ and

prefers a monolayer with zero curvature ($H_0 = 0$). In contrast, LPC comprising only one lipophilic fatty acid and a polar head group exhibits $p < 1$ and its optimum curvature is convex ($H_0 < 0$). Convex curvature of LPC is important for the formation of small oil droplets and the stabilisation of marine PL o/w emulsions.

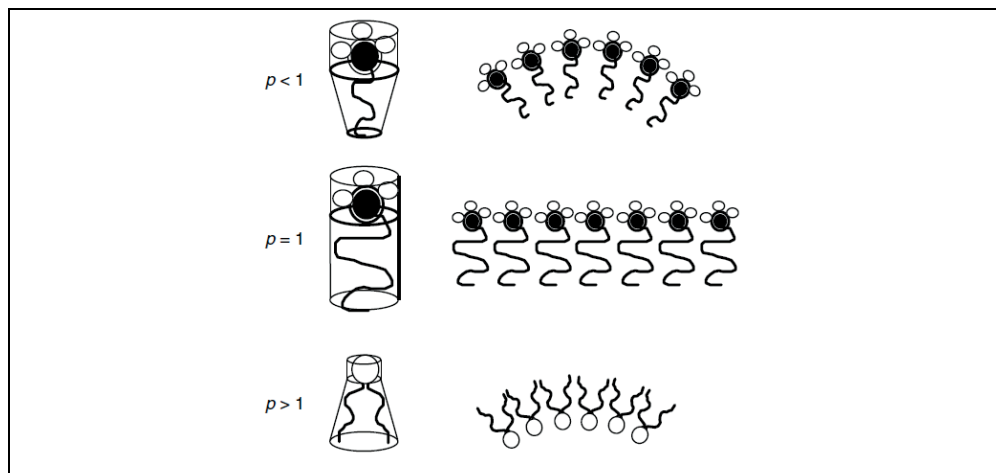


Figure 3.3 The physico-chemical properties of surfactants can be related to their molecular geometry (Adapted from McClements, 2005).

3.4 Factors that influence lipid oxidation in emulsions

The mechanism of lipid oxidation in the o/w emulsion is different from the bulk oil system. This is because an o/w emulsion has an aqueous phase which contains both prooxidants and antioxidants, and an oil-water interface where the interactions between oil phase and prooxidants in aqueous phase may be enhanced (McClements & Decker, 2000). Some studies (Cercaci et al., 2007; Chee et al., 2006) reported that the lipid is oxidized faster in o/w emulsions than bulk oil. This is because the emulsification process itself might promote oxidation and the presence of interfacial phases in o/w emulsions might also increase the interactions between lipid phase and prooxidant compounds in aqueous phase. On the contrary, other studies (Belhaj et al., 2010; Garcia et al., 2006) reported that emulsification improved the oxidative stability of n-3 fatty acids oils due to the possibilities of using a) hydrophobic antioxidant which were more efficient in emulsions system, b) emulsifiers such as maltodextrin or phospholipids which have antioxidative properties. As shown in Table 3.3,

several factors may affect the lipid oxidation in o/w emulsions as suggested by Waraho and co-workers (2011).

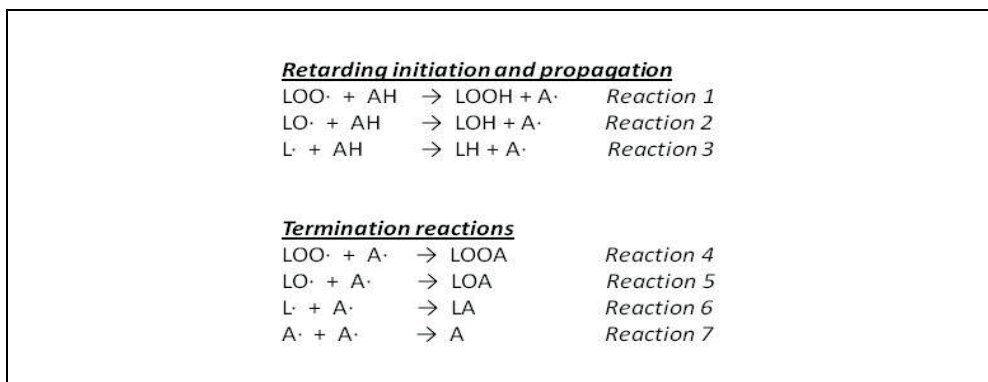
In the present Ph.D. study, only the main factors (the presence of prooxidant and antioxidant in marine PL) that influence lipid oxidation in marine PL emulsions are discussed in detail. This is because marine PL were found to contain prooxidant impurities (free fatty acids, hydroperoxides, transition metals, etc.) and antioxidative compounds (polar head group of phospholipid itself, α -tocopherol, pyrroles, residues of amino acids/protein, etc.) that might influence the oxidative stability of marine PL emulsions. Discussion on the oxidative stability of PL can be found in Chapter 2, whereas the antioxidative properties of pyrroles can be found in Chapter 4.

Table 3.3 Factors capable of inhibiting lipid oxidation in oil-in-water emulsions. Adapted from Waraho et al (2011).

Characteristic	Property	Factors
Lipid Phase	Composition	<ul style="list-style-type: none"> Degree of unsaturation Proxidant impurities, e.g., free fatty acids, hydroperoxides Inherent antioxidants, e.g., free radical scavengers and chelators Added antioxidants e.g., free radical scavengers and chelators
	Physical state-solid fat content and crystal properties	<ul style="list-style-type: none"> Solubility, partitioning and diffusion of antioxidants and prooxidants Rheology determines diffusion of antioxidants and prooxidants Polarity determines partition coefficients.
	Physical properties	
Aqueous Phase	Composition - pH, ionic strength, solutes	<ul style="list-style-type: none"> Prooxidant impurities, e.g., transition metals, photosensitizers, enzymes Inherent antioxidants, e.g., chelators, free radical scavengers Added antioxidant e.g., chelators, free radical scavengers Micelles may alter location of antioxidants and prooxidants Reducing agents that can redox cycle prooxidant metals Solubility, partitioning and diffusion of reactants and products
	Physical state - ice crystal structure and location	
	Physical properties	<ul style="list-style-type: none"> Rheology determines diffusion of antioxidant and prooxidants Polarity determines partition coefficients
Interfacial Phase	Composition	<ul style="list-style-type: none"> Anti-prooxidant activity Impurities (hydroperoxides)
	Thickness	<ul style="list-style-type: none"> Steric hindrance of interactions between water and oil soluble components
	Charge Permeability	<ul style="list-style-type: none"> Electrostatic attraction/repulsion of antioxidants and prooxidants Diffusion of antioxidants and prooxidants in lipid and aqueous phase
Structural Organization	Emulsion	<ul style="list-style-type: none"> Droplet concentration Droplet size distribution (surface area and light scattering) Porosity Exposed lipid levels Emulsion droplet characteristics upon rehydration Hydrogel composition, structure and properties
	Spray dried powder	
	Hydrogel particles	

3.4.1 Effect of antioxidant toward lipid oxidation in marine PL emulsions

Incorporation of antioxidants into marine PL emulsions is expected to be one of the effective methods to retard lipid oxidation. However, there are several factors that may impact the activity of antioxidants such as the concentration of antioxidant, partitioning between oil, aqueous and interfacial phases, interactions with other food components, pH, ionic strength, temperature, etc (Frankel, 2005). In emulsion, antioxidants inhibit lipid oxidation through a) scavenging free radicals by primary antioxidant; b) inactivating prooxidants by secondary antioxidant. Examples of primary antioxidants include phenolic compounds, α -tocopherol, ascorbate, and some synthetic free radical scavengers such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tertiary butylhydroquinone (TBHQ), etc. As shown in Scheme 1 (reactions 1-7), primary antioxidants (AH) inhibit lipid oxidation by interfering the chain propagation and initiation through donation of a hydrogen to free radicals such as lipid peroxy radical (LOO^\bullet), lipid alkoxy radical (LO^\bullet) and lipid alkyl radical (L^\bullet) to form stable non radical products. The formation of stable non radical products and less reactive antioxidant radicals (A^\bullet) is important as these reactions inhibit further decomposition of lipid radicals into aldehydes. In addition, the antioxidant radicals can further scavenge free radicals by participating in the termination of oxidation. The reaction mechanism between antioxidant and lipid radicals is shown in Scheme 3.1.



Scheme 3.1 Overview of antioxidant reactions with lipid radicals and other antioxidant radicals. AH: antioxidant; LOO^\bullet : lipid peroxy radical; LO^\bullet : lipid alkoxy radical; L^\bullet : lipid alkyl radical; LOOH: lipid hydroperoxides; LOH: lipid alcohol; LH: lipid; A^\bullet : antioxidant radical; LOOA, LOA and LA: lipid conjugates with antioxidant and AA: antioxidant dimer. Adapted from Chaiyasit et al. (2007).

In contrast, the secondary antioxidants retard lipid oxidation through several mechanisms without converting the free radicals into more stable products. These mechanisms include chelation of transition metals, oxygen scavenging, and singlet oxygen quenching. Examples of chelators include citric acids, phosphoric acid and ethylenediaminetetraacetic acid (EDTA).

In contrast to the bulk oil systems, the ability of antioxidant to inhibit the lipid oxidation in emulsion depending on its physical location, either in oil, aqueous or interfacial regions (Koga & Terao, 1995). According to the polar paradox hypothesis, non-polar antioxidants are more effective in o/w emulsions. This is because non-polar antioxidants are retained in the emulsion droplets or accumulate at oil-water interface, where oxidation is most prevalent. For instance, non-polar antioxidants such as α -tocopherol, ascorbyl palmitate, carnosol are more effective than their polar counterparts such as Trolox, ascorbic acid and carnosic acid in emulsions as reported by several studies (Frankel et al., 1996a; Frankel et al., 1996b; Chaiyasit et al., 2007). However, recent studies (Yuji et al., 2007; Sasaki et al., 2010) showed that antioxidant polar paradox hypothesis does not apply to all compounds and one of the reasons is due to the 'cut-off effect' hypothesis. According to this hypothesis, the antioxidant capacity of lipophilized compound such as chlorogenic acid in o/w emulsions increases as its esterified alkyl chain length increases to a certain level. The further increase of the esterified alkyl chain length might decrease the antioxidant capacity of lipophilized compounds due to the micellization (Laguerre et al., 2009). In addition, antioxidants can interact with other compounds in real food systems. For instance, phenolic antioxidants, ascorbic acid and carotenoids can reduce transition metals and thus promote lipid oxidation (Jacobsen et al., 2001; Sørensen et al., 2008; Boon et al., 2009).

3.4.2 Effect prooxidants toward lipid oxidation in marine PL emulsions

The presence of prooxidants such as trace hydroperoxides, transition metals and free fatty acids may promote lipid oxidation in o/w emulsion prepared from marine PL. The prooxidant activity of free fatty acids is most likely due to their ability to increase the negative charge of the emulsion droplets and thus increase metal-lipid interactions (Waraho et al., 2011). Several studies (Mei et al., 1998a; 1998b; Minotti & Aust, 1989) suggested that the interactions between lipid hydroperoxides, which are located at the surface of droplets with the transition

metals originating in the aqueous phase is the most common cause of lipid oxidation. For instance, a study of Mozuraityte and co-workers (2006a) showed that the lipid oxidation rate as measured by oxygen consumption increased immediately in liposome dispersion prepared from cod phospholipids after addition of transition metal, ferrous ions (Fe^{2+}). This phenomenon is due to the fast fixation of Fe^{2+} to the negative surface charge of PL liposomes. They also reported that addition of HCl or NaCl reduced the connection between Fe^{2+} and liposomes and thereby decreased the lipid oxidation in liposome dispersion. The presence of transition metals such as ferrous and ferric ions (Fe^{2+} and Fe^{3+}), primarily promote lipid oxidation by decomposing lipid hydroperoxide into free radical via a Fenton-type reaction as suggested by Dunford (1987). Thus, lipid oxidation could be greatly suppressed when the level of hydroperoxides was reduced in model system as suggested by Tadolini & Hakim (1996). In addition, the type, concentration and chemical state of transition metal may influence the decomposition rate of hydroperoxides differently. For instance, ferrous ion is a stronger prooxidant than ferric ion due to its higher solubility and reactivity (Halliwell & Gutteridge, 1990). As shown in Figure 3.4, transition metals decompose hydroperoxides (LOOH) to form alkoxy radical (LO^\bullet) and peroxy radicals (LOO^\bullet), which can then abstract further H atoms. Free radicals (L^\bullet) can then react with triplet oxygen to form peroxy radicals. In addition, transition metals can also abstract H from unsaturated lipid (LH) to form free radical (L^\bullet), but this reaction is relatively slow and therefore is not an important pathway of lipid oxidation (Reische et al., 1998).

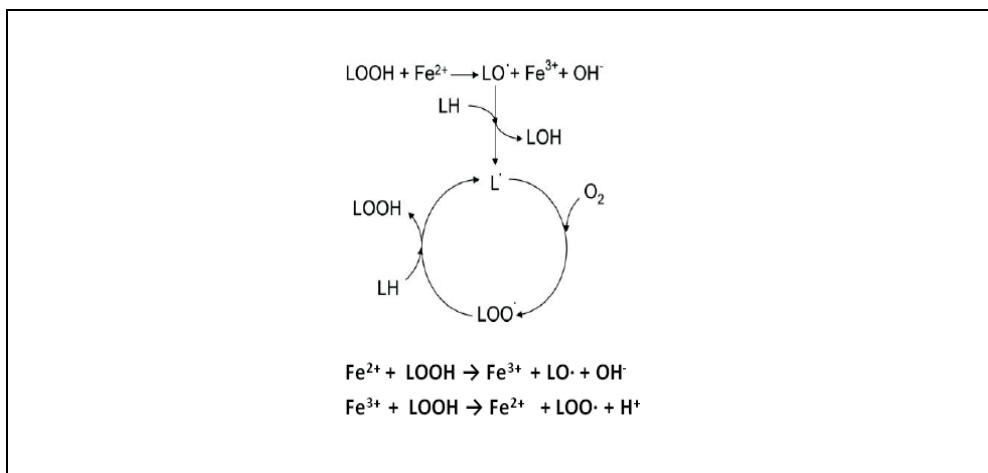


Figure 3.4 Lipid oxidation mechanisms by transition metals (Adapted from Mozuraityte et al., 2006a; Berger and Hamilton, 1995; Reische et al., 1998).

CHAPTER 4 NON-ENZYMATIC BROWNING IN MARINE PL

In this chapter, non-enzymatic browning reactions are discussed with special emphasis on browning reaction as a consequence of lipid peroxidation. It is speculated that occurrence of browning reaction in marine PL is mainly due to lipid peroxidation. However, the Maillard reaction is included for comparison as it is a well known reaction in non-enzymatic browning reactions. Furthermore, browning reactions may occur in marine PL as a result of Maillard reaction and this was confirmed by the detection of minor quantity of reducing sugar in marine PL used in the present Ph.D. study (through ^{13}C NMR in a preliminary experiment).

Non-enzymatic browning reactions occur in food systems as a consequence of a) Maillard reaction, b) lipid peroxidation, c) caramelization, d) oxidation of ascorbic acid (Reineccius 2006). Maillard reaction is the most common non-enzymatic browning. Maillard pathway is initiated by the primary reaction of the reactive carbonyl group of a sugar with the nucleophilic amino group of an amino acid. However, sugar or carbohydrates are not the only source of reactive carbonyls in foods. Lipid oxidation also produces reactive α -dicarbonyls that contribute to non-enzymatic browning reactions. From a chemical point of view, lipid derived reactive carbonyls should also behave like reducing sugars and they are able to react with the nucleophilic amino group of amino acids to produce an analogous cascade of reactions (Zamora & Hidalgo, 2011).

Among these four reactions mentioned above, the Maillard reaction and lipid peroxidation are known to be interrelated (Hidalgo & Zamora, 2005). These two reactions follow similar reaction pathways and produce common intermediates and carbonyl derivatives. For instance, two dicarbonyl compounds are produced from both oxidized lipids and carbohydrates and therefore the so-called Strecker degradation of amino acids by dicarbonyl compounds can be initiated either by Maillard reaction pathway or lipid peroxidation pathway through a similar mechanism. In the present Ph.D. study, non-enzymatic browning mainly produced as a consequence of lipid oxidation. Therefore, non-enzymatic browning produced as a consequence of Maillard reaction is briefly discussed.

4.1 Non-enzymatic browning produced as a consequence of lipid oxidation

Lipids play an important role in non-enzymatic browning due to the high reactivity of secondary lipid oxidation products, namely α , β -unsaturated aldehydes or dicarbonyl (malonaldehydes) with primary amine group (Pokorny & Sakurai, 2002; Thanonkaew et al., 2006b). A simplified scheme explaining the mechanism of non-enzymatic browning in the lipid oxidation pathway is shown in Figure 4.1. Firstly, lipid is oxidized to produce hydroperoxides, which are relatively unstable and may further decompose to form a wide range of secondary oxidation products such as aldehydes, ketones, alcohols, epoxides and hydrocarbons acids. These lipid oxidation products may polymerize among themselves to produce brown colored oxypolymers (Khayat and Schwall, 1983). However, the primary oxidation products or their degradation products, namely unsaturated and polyunsaturated aldehydes may also react with the primary amine groups of phosphatidylethanolamine, amino acids or protein to form highly colored polymers/pyrrole polymers through aldol condensation or carbonyl-amine polymerization, which contributes to non-enzymatic browning reactions (Hidalgo & Zamora, 1993; Pokorny & Sakurai, 2002; Thanonkaew et al., 2006b). In general, lipid oxidation products contribute to non-enzymatic browning through formation of colored pyrrole polymers and Strecker degradation of amino acids.

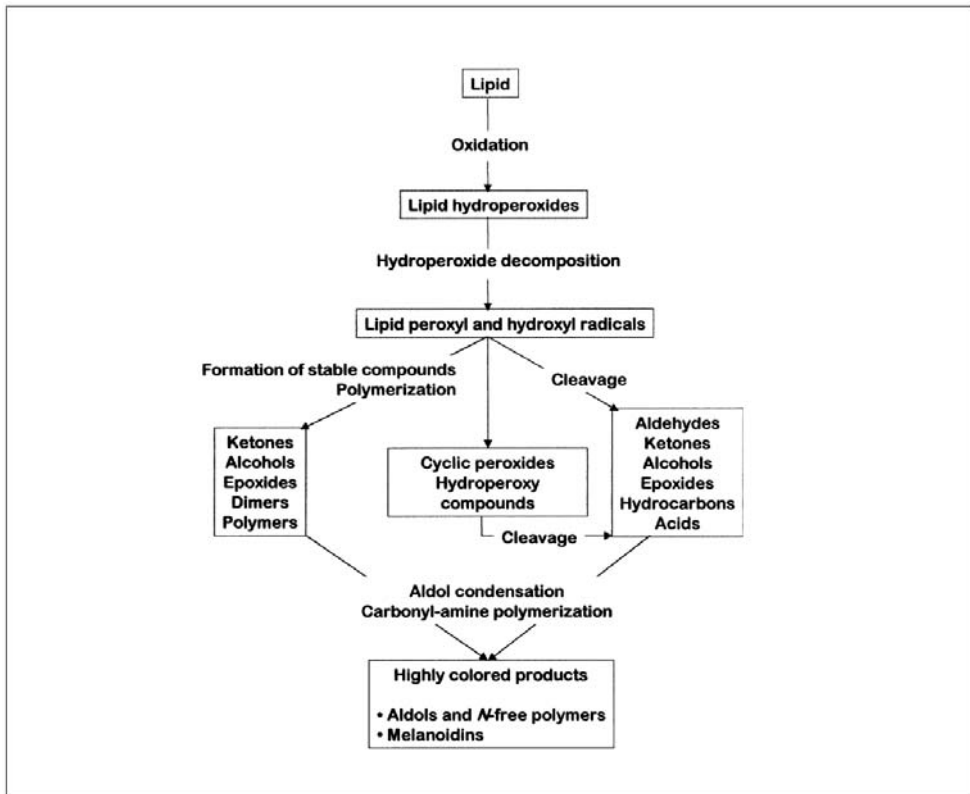


Figure 4.1 Non-enzymatic browning produced as lipid oxidation pathway (Zamora & Hidalgo, 2005).

4.2.1 Strecker degradation

Strecker degradation (SD) is a minor pathway in non-enzymatic browning and involves the oxidative deamination of α -amino acids in the presence of compound such as reducing sugars, lipid oxidation products, dehydroascorbic acid or other Strecker reagents. In the Maillard reaction pathway, it involves an initial Schiff base formation of an amino acid with an α -dicarbonyl derivatives from carbohydrate or sugar. After rearrangement, decarboxylation and hydrolysis, an α -aminoketone and an aldehyde (containing one carbon atom less than the original acid) usually known as Strecker aldehyde are produced (as shown in Figure 4.2a). α -aminoketone are precursors for important food flavor compounds such as pyrazines, oxazoles and thiazoles. In addition, α -aminoketone may undergo self-condensation

or condensation with other aminoketone to form alkylpyrazines (Hidalgo & Zamora, 2005; Baynes et al., 2005).

Lipid oxidation also produces α -dicarbonyl derivatives analogous to that of carbohydrates and therefore is involved in Strecker degradation (Hidalgo & Zamora, 2005; 2008; Zamora & Hidalgo, 2011). For instance, tertiary lipid oxidation products such as unsaturated epoxy keto fatty esters, epoxyalkenals and hydroxyalkenals can degrade amino acids through SD reaction. In the lipid peroxidation pathway, SD reaction follows a similar mechanism to that of SD in Maillard reaction pathway. Firstly, an imine is produced and it undergoes rearrangement, decarboxylation, hydrolysis and subsequently evolution into a Strecker aldehyde and a hydroxyl amino compound (as shown in Figure 4.2b). Hydroxyl amino compounds are responsible for the formation of 2-alkylpyridines in this reaction.

Hidalgo and Zamora (2004) reported that 4,5-epoxy-2-alkenals, namely, 4,5 (*E*)-epoxy-2(*E*)-decenal and 4, 5 (*E*)-epoxy-2(*E*)-heptenal degraded phenylalanine to form a Strecker aldehyde phenylacetaldehyde and 2-alkylpyridine at 37 °C in addition to pyrroles formation. 2-ethylpyridine was produced from 4, 5 (*E*)-epoxy-2(*E*)-heptenal and 2-pentylpyridine was produced from 4, 5 (*E*)-epoxy-2(*E*)-decenal (as shown in Figure 4.3). Similar to Maillard reaction, epoxyalkenals can also convert amino acids into corresponding α -keto acids depending on the reaction conditions, in addition to Strecker mechanism as mentioned earlier (Zamora et al., 2006). Furthermore, it is suggested that the presence of two oxygenated function groups in the tertiary lipid oxidation products, namely one carbonyl group and one epoxy or hydroxyl group is required for the SD reaction to occur. In addition, secondary lipid oxidation products such as alkadienals and ketodienes can also degrade amino acids to their corresponding Strecker aldehydes when secondary lipid volatiles are further oxidized under appropriate conditions (Zamora et al., 2007).

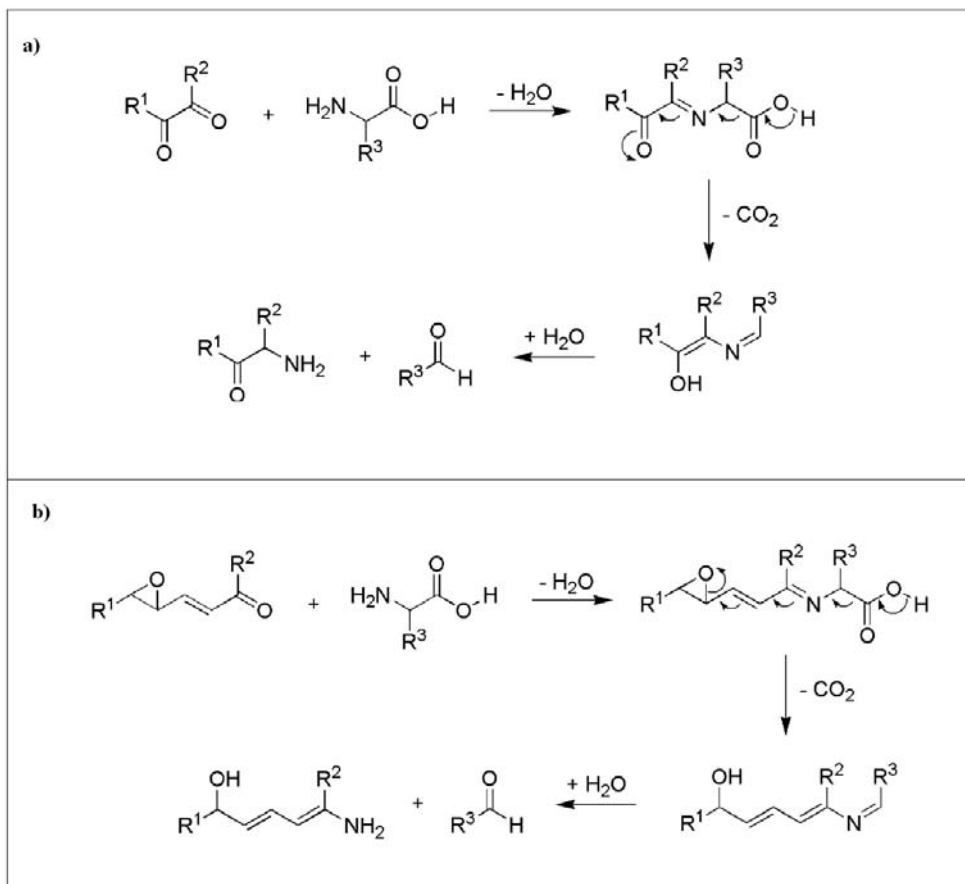


Figure 4.2 a) Strecker degradation of amino acids produced by α -dicarbonyl compounds in the Maillard reaction pathway; b) Strecker degradation of amino acids produced by 4, 5-epoxy-2-alkenals in the lipid peroxidation pathway (Adapted from Hidalgo & Zamora, 2005).

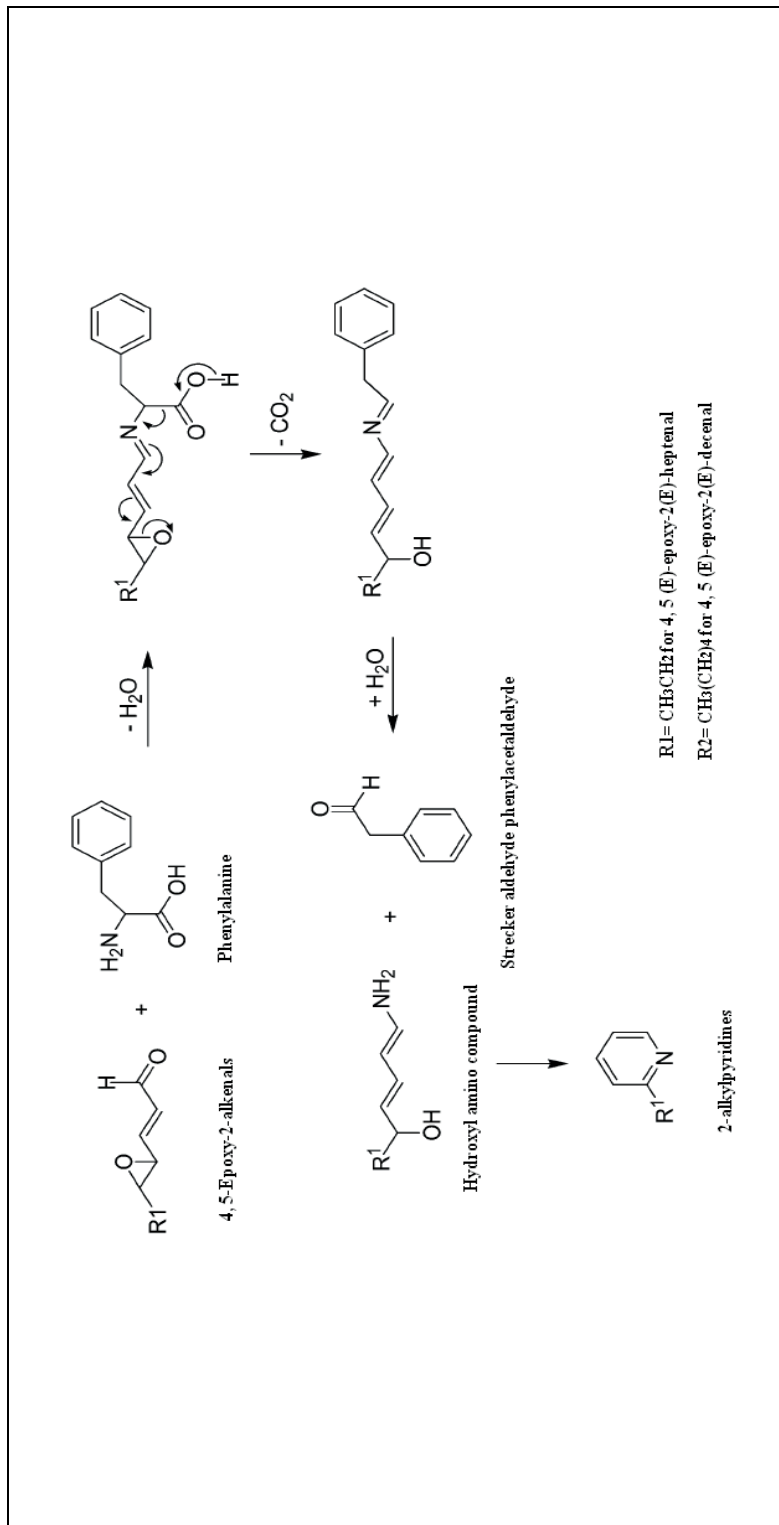


Figure 4.3 Strecker degradation of phenylalanine by 4, 5-epoxy-2-alkenals (Hidalgo and Zamora 2004).

4.2.2 Pyrroles formation and polymerization

In addition to Strecker degradation, the carbonyl derivatives from oxidized lipids participate in pyrroles formation and polymerization. Currently, there are 2 main proposals for the mechanisms contributing to melanoidin formation: aldol condensation and pyrrole polymerization. The first mechanism for non-enzymatic browning as a consequence of lipid oxidation was proposed by Mohammad et al (1949), which was a repeated aldol condensation. According to Mohammad and co-workers, the carbonyl derivatives from unsaturated lipids condense with the free amine group from protein to form imino Schiff base. Then, Schiff bases polymerize through aldol condensation to produce dimers and melanoidin like macromolecules. These polymeric brown materials are not stable and cause generation of new volatiles through scission of the macromolecules or dehydration. However, a more recent mechanism based on the polymerization of the *N*-substituted hydroxyalkylpyrroles was proposed by Hidalgo and Zamora (1993) for non-enzymatic browning. The detail of this mechanism is stated as follows:

In lipid peroxidation pathway, tertiary lipid oxidation product such as 4, 5-epoxy-2-alkenals firstly react with the amine groups of amino acids, proteins and amino phospholipid to produce an imine, which then evolves into a cyclic intermediate. This intermediate subsequently is converted into two different pyrrole derivatives and a short chain aldehyde depending on the reaction conditions, namely 2-(1-hydroxyalkyl)pyrroles and *N*-substituted pyrroles. Formation of 2-(1-hydroxyalkyl)pyrroles is always accompanied by a formation of *N*-substituted pyrroles (Zamora & Hidalgo 1994; 1995). As far as the stability is concerned, *N*-substituted pyrroles are stable ALEs. In contrast, 2-(1-hydroxyalkyl) pyrroles are unstable and they polymerize spontaneously to form melanoidin/lipofuscin-like macromolecules (as shown in Figure 4.4). Polymerization occurs by successive dehydrations between the polymers and the monomers, and may also include other pyrroles. In fact, pyrroles formation and polymerization are responsible for the browning development in the systems containing both carbonyl derivatives and primary amine group (Zamora et al., 2000; 2004). Zamora et al (2000) reported that a high correlation was obtained among the measurements of color, fluorescence and pyrrolization in 4,5(*E*)-epoxy-2-(*E*)-heptenal/lysine and linolenic acid/lysine model systems after incubation at 37 °C and 60 °C. The color and fluorescence production in these model systems was due to the pyrrole formation and polymerization. In addition, Zamora et al (2004) also showed that pyrrolization of PL contributed to the oil darkening in

poorly degummed edible oils, refined olive and soybean oils. In addition, according to Uematsu and co-workers (2002), the increase in degree of unsaturation of lipids also led to the increase in non-enzymatic browning reactions.

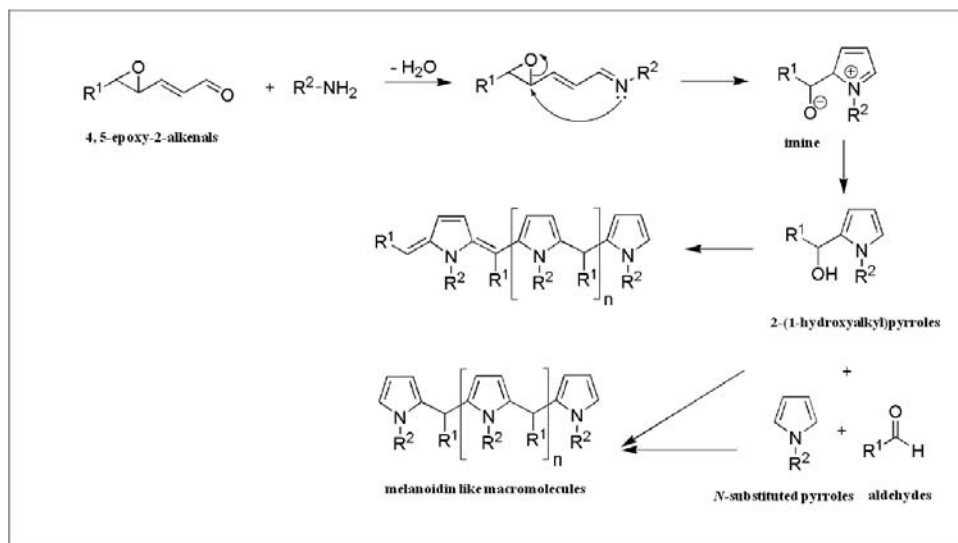


Figure 4.4 Pyrroles formation and polymerization in lipid peroxidation pathway (Adapted from Hidalgo & Zamora 2005).

4.2.3 Antioxidative properties of pyrroles

Pyrroles formed between oxidized lipids and the amine groups of protein/amino acids were shown to have antioxidative properties. Several studies reported that naturally formed antioxidative pyrroles from oxidized lipid/amino acid reaction are able to protect bulk vegetable oils against oxidation (Alaiz et al., 1995a; 1995b; 1996) or delay the peroxidative process initiated in a soybean oil at the same time that they were being produced (Alaiz et al., 1995c). Furthermore, the presence of the antioxidative compound, namely pyrrole was confirmed by GC-MS (Alaiz et al., 1996) and the reaction mechanism for pyrroles formation is well characterized (Hidalgo & Zamora 1993, Zamora & Hidalgo 1995). However, the antioxidative activity of pyrroles produced during the oxidative process was greatly increased with the addition of artificial antioxidants such as BHT or α -tocopherol (Ahmad et al., 1998) or decreased due to the pyrrole polymerization (Anese & Nicoli, 2003; Manzocco et al., 1998). For instance, slightly browned samples were reported to be more antioxidative than

samples in which non-enzymatic browning has been highly developed due to the polymerization. The effect of pyrrole polymerization on the antioxidative activity of non-enzymatic browning reactions was well studied by Hidalgo and co-workers (2003). In the first part of this study, they investigated the antioxidative activities of eight different pyrroles. According to their findings, antioxidative activity exhibited by pyrroles could be categorized into 3 main groups and was in the order stated as follows: a) pyrroles with no free α position > pyrroles with free α position > pyrroles with an oxygenated group. In other words, the antioxidative activity of pyrrole derivatives was in the order stated as follows: 1, 2, 5-trimethylpyrrole & 2, 5-dimethylpyrrole > pyrrole & 1-methylpyrrole > 2-acetylpyrrole, 2-acetyl-1-methylpyrrole, pyrrole-2-carboxaldehyde & 1-methyl-2-pyrrolicarboxaldehyde. The structures of these molecules are shown in Figure 4.5. In the second part, they investigated the changes in antioxidative activity during the polymerization of 2-(1-hydroxyethyl)-1-methylpyrrole (HMP). They reported that HMP firstly produced dimers (DIM), consequently both HMP and DIM polymerized to produce trimers (TRI), tetramers (TET) and higher polymers. They also reported that polymerization produced mainly the DIM rather than the higher polymers. In addition, polymerization contributed to the development of yellow color. As the incubation progressed, these DIM were transformed into polymers, and therefore their antioxidative activity decreased. Furthermore, DIM were found to be 2.5 times more antioxidative than HMP. Dimers contained two pyrrole rings without oxygenated functions and one of them having no free α -position. In summary, their studies showed that the antioxidative activity observed in a non-enzymatic browning reaction is the sum of the antioxidative activities of the different compounds present in the samples. Thus, antioxidative activity of a non-enzymatic browning reaction might change at the same time when the different pyrroles are either produced or evolved into polymers.

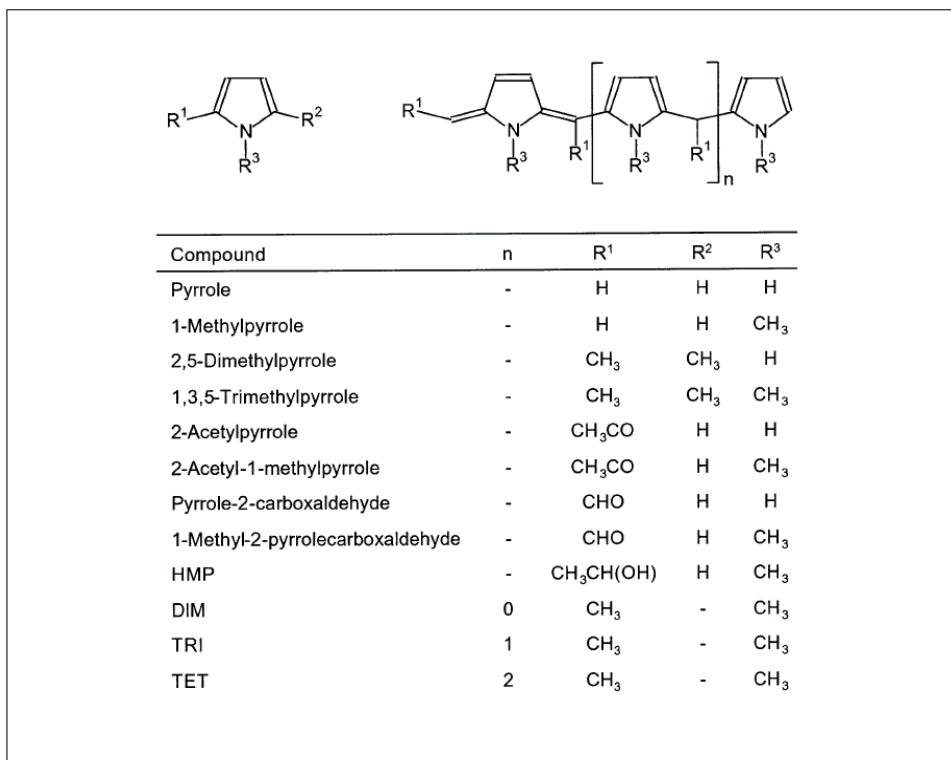


Figure 4.5 Structures of the different pyrrole derivatives. HMP=2-(1-hydroxyethyl)-1-methylpyrrole, DIM=dimers, TRI=trimers, TET=tetramers (Adapted from Hidalgo et al., 2003).

4.2.4 Antioxidative activity of pyrroles in oxidized PL

More recent studies on pyrroles particularly focusing on antioxidative activity of pyrroles in oxidized phospholipids (PL) were reported by Hidalgo and co-workers (2005b; 2006; 2007). Hidalgo and co-workers (2005b) investigated the antioxidative activities of native and oxidized soybean phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) in protection of soybean oil heated in darkness under air at 60 °C. They reported that the slightly oxidized PE was more antioxidative than the native PE due to the pyrroles formation in pyrrolized PE. The oxidized PL without an amine group such as PC and PI were less antioxidative than their native form as they did not produce pyrroles while they were being consumed during the oxidation. In 2006, they further investigated the antioxidative activity of PE, PC, lysine (Lys) and their mixtures in refined olive oil (Hidalgo et al., 2006). A summary of their findings is stated as follows:

a) Addition of PE or Lys alone increased the induction periods (IPs) of refined olive oil, whereas PC did not show any protective effect against lipid oxidation. The protective effect provided by PE or Lys alone or their mixtures could be ascribed to formation of pyrroles, which had antioxidative properties as mentioned earlier. b) A mixture of PE/Lys or PC/Lys exhibited a synergistic effect. This synergistic effect was highest when 300 ppm of PE and 100 ppm of Lys were used. This is because a higher concentration of easily oxidizable lipids was more important than a higher concentration of the primary amine group from Lys. In PE/Lys system, two identical groups of pyrroles with different properties were produced depending on the reaction of oxidized lipids either with PE or with Lys, those produced by PE were lipophilic and those produced by amino acids were hydrophilic (as shown in Figure 4.6). The finding is in accordance with the findings of their other studies (Zamora et al., 2005; Hidalgo et al., 2005b). In addition, they reported that hydrophilic antioxidants produced by Lys were more effective in protecting olive oil, which could be an explanation for a high protective effect shown by PC/Lys system despite only one type of pyrroles (hydrophilic pyrroles) was formed in this system. A mixture of PC/PE did not exhibit any synergism due to the absence of amino acid and only lipophilic pyrroles were formed in this system. Lipophilic pyrroles were less effective in bulk oil system.

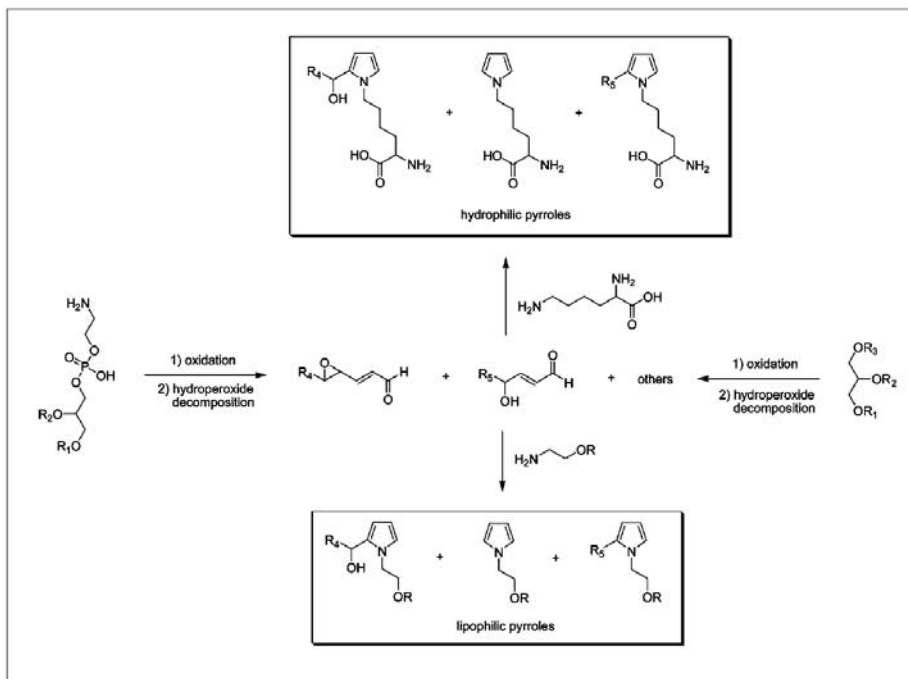


Figure 4.6 Production of reactive carbonyls during PE and triacylglycerol oxidation and the later formation of pyrrolized phospholipids or amino acids by carbonyl-amine reactions (Adapted from Hidalgo et al., 2006).

4.2.5 Effect of tocopherol on the antioxidative activity of pyrroles

Hidalgo and co-workers (2007) investigated the effect of tocopherol on antioxidative activity of pyrroles produced in slightly oxidized PE, PC Lys or their mixtures in tocopherol stripped olive oil. Their findings showed that antioxidative activity of pyrroles might be greatly increased with the addition of tocopherol. For instance, addition of PE or Lys together with α -tocopherol increased the induction period of olive oil. Furthermore, a mixture of PE/Lys or PC/Lys is more effective than PC/PE mixture to protect the olive oil with addition of α -tocopherol.

4.3 Non-enzymatic browning in marine PL liposomes

Studies on non-enzymatic browning in marine PL system are scarcely available in literature. Studies on non-enzymatic browning in marine PL liposome have recently been reported by Thanonkaew et al (2005; 2006a; 2006b; 2007). Thanonkaew and co-workers (2006b) investigated the non-enzymatic browning development in squid (*Loligo peali*) lipids and proteins. Their studies suggested that lipid oxidation (as measured by thiobarbituric acid reactive substance, TBARS) increased simultaneously with yellowness (as measured by b^* values) and pyrroles content, and decreased concomitantly in free amines when squid microsomes, squid PL liposomes and egg yolk lecithin liposomes were oxidized with iron and ascorbate. They also reported that the occurrence of non-enzymatic browning in squid muscle could primarily be ascribed to the reaction between the amine groups of PE and aldehydic lipid oxidation products. Furthermore, non-enzymatic browning was found to be higher in squid PL liposomes than egg yolk lecithin liposomes due to the higher degree of unsaturation in squid lipid (Thanonkaew et al., 2006b). When egg yolk lecithin liposomes were incubated with different aldehydic lipid oxidation products at 37°C for 15 hours, they reported that the saturated aldehydes, namely propanal and hexanal had the least impact on yellowness and chemical properties of liposomes. In contrast, the monounsaturated aldehydes especially *trans*-2-heptenal, *trans*-2-octenal and *trans, trans*-2, 4-hexadienal changed significantly ($p < 0.05$) the yellowness, free amines and pyrroles content of liposomes. In addition, they also investigated lipid oxidation, yellowness, loss of amine groups, and pyrroles content in the liposome systems prepared from cuttlefish in the presence of FeCl_3 and ascorbic acid (Thanonkaew et al., 2007). Their study suggested that the increase of incubation temperature from 0 to 37 °C or incubation time from 0 to 24 hour led to the increase of TBARS and the b^* value of cuttlefish liposomes with a coincidental decrease in amine groups. Furthermore, pyrrolization was found to increase over time as lipid oxidation and yellowness development proceeded in cuttlefish liposome in addition to the loss of amine groups. Their study also showed that FeCl_3 and ascorbic acid had pro-oxidative and concentration dependent effect in cuttlefish liposomes, whereas sodium chloride (0-2%) had anti-oxidative effects toward lipid oxidation and non-enzymatic browning in the liposomes. In general, this study also suggested a positive correlation between lipid oxidation and non-enzymatic browning development in cuttlefish PL. This finding is in agreement with the findings of their previous study.

The experimental work in this thesis was carried out as described in paper II to VI. The results and discussion of the experimental work are divided into 4 major parts, and also be drawn on the theoretical background as reported in the review paper (I): *Part 1*: evaluation of physico-chemical properties of marine PL emulsions (paper II), *Part 2*: evaluation of oxidative stability in marine PL emulsions (paper III & IV), *Part 3*: evaluation of non-enzymatic browning reactions in marine PL emulsions (paper III & V), and *Part 4*: evaluation of oxidative stability and sensory properties of marine PL fortified foods (paper VI). Figure 5.1 presents a schematic overview of the present Ph.D. study, including the related papers (I-VI) found in the appendix section. A dotted line square over part 2 and part 3 indicates that investigation for these two parts were carried out simultaneously in paper III and V.

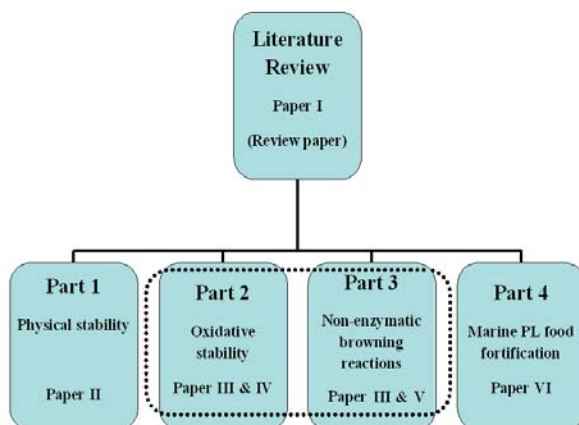


Figure 5.1: A schematic overview of the present Ph.D. study.

5.1 An overview of marine PL preparations used in the present Ph.D. study

A total of six commercial marine PL preparations were used to prepare emulsions or marine PL dispersions. The details of these marine PL preparations are shown in Table 5.1 and the relevant specifications/data sheets can be found in appendix.

Table 5.1: An overview of marine PL preparations used in the present Ph.D. study

Name	MPT	MPL	LC	MPW	MPN	MGK
Used and reported in paper	Paper II	Paper II & III	Paper II, III & V	Paper III, IV & V	Paper VI	Paper VI
Suppliers	University of Tromsø	Triple Nine, Denmark	PhosphoTech, France	Triple Nine, Denmark	Triple Nine, Denmark	Polaris, France
Brand Name	CAVIAR PHOSPHOLIPIDS	999MPL40	LC60	999MPL40	999MPL40	MEGAKRILL OIL
Sources	salmon roe	sprat fish meal	fish by products	sprat fish meal	sprat fish meal	antarctic krill <i>Euphausia superba</i>
Total EPA & DHA (% area GC)	30.00	29.10	24.31	28.50	32.80	28.00
Phosphatidylcholine PC (%)	24.74	18.90	20.87	18.30	16.14	32.0 (including LPC)
Phosphatidylethanolamine PE (%)	3.01	6.00	6.11	4.70	4.50	4.00
Phosphatidylinositol PI (%)	0.51	2.50	0.96	2.10	1.84	2.00
Sphingomyelin SPM (%)	-	-	1.59	-	3.50	-
Lysophosphatidylcholine LPC (%)	0.17	2.40	3.47	3.40	5.37	ND
Other phospholipids*	-	10.30	-	8.90	12.99	2.00
Total phospholipid (%)	28.43	40.10	43.84	41.50	44.34	40.00
Triglycerides, TAG (%)	48.00	40.00	1.00	40.00	33.00	ND
Cholesterol, CHO (%)	5.00	3.00	15.00	2.00	3.00	ND
Free fatty acids, FFA (%)	3.50	17.00	21.00	16.00	20.50	ND
α -Tocopherol (mg/Kg)	341.10	94.20	1464.20	73.40	144.00	466.00
Esterified astaxanthin (mg/Kg)	18.80	-	-	-	-	50.00
Ethoxyquin (mg/Kg)	-	108.70	-	<10.00	<10.00	-
Transition metal, iron (ppm)	1.85	25.75	2.01	20.08	6.56	<1
Peroxide Value (meq/kg)	3.48±0.51	1.86±0.78	1.75±0.09	0.81±0.04	1.11±0.01	1.07±0.01

(-) = Not detectable, ND = Not determined. *Other phospholipids might include APE, LPE, glycolipids, etc.

In paper II, III and VI, emulsions were prepared either solely from marine PL or from a mixture of marine PL and fish oil. In paper IV and V, liposomal dispersions were prepared from purified marine PL and authentic PL standards. The details of each part will be further discussed later. In terms of marine PL manufacturing process, only limited information was obtained as this information was confidential to some of the manufacturers. To the best of our knowledge, LC was extracted from fish by-products at low temperature by using enzymatic hydrolysis, whereas MPT was extracted from salmon roe by using ethanol at a maximum temperature of 60 °C and all marine PL preparations from Triple Nine were extracted from fish meal by using hexane. In addition, fish meal was produced at high temperature (90 - 100 °C). Marine PL preparation, MGK was extracted from Antarctic krill *Euphausia superba*. The fish oil of high quality (Maritex 43-01) was used for emulsions preparation and it was obtained from Maritex A/S (subsidiary of TINE). This fish oil had low initial PV (0.16 meq/kg) and comprised 240.0 mg/kg α -tocopherol, 99.3 mg/kg γ -tocopherol and 37.9 mg/kg δ -tocopherol. The total of EPA and DHA in this fish oil was approximately 20.84 (% area GC). In paper II, three marine PL preparations, namely MPT, MPL and LC were used to investigate the physico-chemical properties of marine PL emulsions. In paper III, two of these marine PL preparations (LC and MPL) were used again, with addition of another marine PL preparation (MPW) to investigate both the oxidative stability and non-enzymatic browning reactions in marine PL emulsions. MPT was not further used in paper III due to its higher initial PV as compared to other marine PL preparations. MPW and MPL had similar chemical composition, except that an additional antioxidant (ethoxyquin) was found in MPL. In paper IV, MPW was purified through acetone precipitation and liposomal dispersions were prepared from the purified marine PL. In paper V, a model study was carried out to further investigate the non-enzymatic browning reaction in marine PL. In this model study, two purified marine PL preparations (from MPW and LC) and two pure authentic PL standards (PC and PE) were used to prepare liposomal dispersions. In paper VI, another two marine PL preparations (MGK and MPN) were used for food fortification. The reasons for choosing these marine PL preparations are discussed in section 5.2.4.

5.2 Experimental approach

5.2.1 Part 1: Evaluation of physico-chemical properties of marine PL emulsions (paper II)

The main objective of this Ph.D. research was to explore the possibility of using marine PL for food fortification. In order to achieve this main objective, the possibility of using marine PL to prepare physically stable emulsions was investigated. This also includes the use of marine PL as emulsifier to prepare physically stable fish oil emulsions. We hypothesized that physico-chemical properties of emulsion could be influenced by the chemical compositions of marine PL preparation used. In order to test this hypothesis, three different commercial marine PL preparations (LC, MPT and MPL) and fish oil (Maritex 43-01) were used to prepare marine PL o/w emulsions. The chemical compositions of all three marine PL were determined prior to the emulsion preparation.

A total of 17 different formulations of marine PL o/w emulsions were prepared through pre-emulsification and homogenization using an Ultra-Turrax followed by a high pressure table homogenizer (as shown in Table 5.2). Firstly, o/w emulsions were prepared using only marine PL. Then, o/w emulsions were prepared using a mixture of marine PL and fish oil at different ratios. Marine PL emulsions were stored in darkness for 32 days at two different storage temperatures; 2°C or room temperature (approx. 20-25° C). The purpose of this storage study was to investigate the effect of temperature towards both physical and oxidative stabilities of marine PL emulsions. In addition to physical stability, a preliminary study of oxidative and hydrolytic stability of marine PL emulsion was done through simple chemical measurements. The oxidative stability of marine PL emulsions was further investigated in part 2 (paper III & IV).

Table 5.2: Experimental design for marine PL emulsions used in paper II

Formulations/ Emulsions	% Fish oil	% Phospholipids			%Total lipids
		MPT	MPL	LC	
MPL2			2.0		2.0
MPL4			4.0		4.0
MPL6			6.0		6.0
MPL8			8.0		8.0
MPL10			10.0		10.0
FMPL05	9.5		0.5		10.0
FMPL1	9.0		1.0		10.0
FMPL2	8.0		2.0		10.0
FMPL3	7.0		3.0		10.0
MPT2		2.0			2.0
MPT10		10.0			10.0
FMPT05	9.5	0.5			10.0
FMPT3	7.0	3.0			10.0
LC2				2.0	2.0
LC10				10.0	10.0
FLC05	9.5			0.5	10.0
FLC3	7.0			3.0	10.0

5.2.2 Part 2: Evaluation of oxidative stability in marine PL emulsions (paper III & IV)

Based on the findings obtained from paper II, two marine PL preparations, namely MPL and LC that gave a high physical and oxidative stability were chosen for emulsion preparation in paper III. We hypothesized that emulsions prepared solely from marine PL are more oxidatively stable than emulsions prepared from a mixture of fish oil and marine PL. In order to test this hypothesis, three different sets of emulsions were prepared from MPL, MPW and LC as shown in Table 5.3. Each set comprises an emulsion prepared solely from marine PL and an emulsion prepared from a mixture of fish oil and marine PL. The received marine PL preparations were used for emulsion preparations without further treatment and therefore these marine PL are termed as ‘untreated marine PL’ in the present Ph.D. thesis. In paper III, the effects of chemical composition and the quality of marine PL toward oxidative stability of marine PL emulsions were investigated.

Marine PL emulsions were stored in darkness at 2 °C for 32 days. Storage at room temperature was discontinued as it adversely affected the oxidative stability of marine PL. In addition, due to the presence of amino acids residues, protein and reducing sugar in marine PL, non-enzymatic browning reactions might occur between the oxidised lipid and the amine group from PE or the amino acids residues. Therefore, the secondary objective of this part was to investigate the non-enzymatic browning reactions as these reactions might affect the lipid oxidation in marine PL emulsions or vice versa. In addition, the composition of residues amino acids of MPL, MPW and LC was determined with the purpose to investigate Strecker degradation of amino acids (SD) as a part of non-enzymatic browning reactions (Table 5.4). The non-enzymatic browning reactions in marine PL were further investigated in paper V (part 3).

Table 5.3: Experimental design for marine PL emulsions used in paper III

*Formulations (in thesis)	Formulations (in paper III)	Fish oil (%)	Phospholipids (%)			Total lipids (%)	Acetate- imidazole buffer (%)
			MPL	MPW	LC		
MPL10	MPL		10.0			10.0	90.0
FMPL3	F-MPL	7.0	3.0			10.0	90.0
MPW10	MPW			10.0		10.0	90.0
FMPW3	F-MPW	7.0		3.0		10.0	90.0
LC10	LC				10.0	10.0	90.0
FLC3	F-LC	7.0			3.0	10.0	90.0

**The sample codes used in the present Ph.D. thesis are different from paper III.*

Table 5.4: List of amino acids residues in marine PL preparations (MPL, MPW and LC).

Marine PL raw materials % (g /100 g marine PL)	MPL	MPW	LC
Amino acids residues			
Leucine	0.01±0.00	-	-
Proline	-	-	3.49±0.40
Alanine	0.09±0.01	0.13±0.01	4.94±0.12
Glycine	0.04±0.00	0.03±0.00	1.04±0.36
Glutamic acid	0.02±0.00	-	0.16±0.07
Isoleucine	0.01±0.00	0.01±0.00	0.14±0.06
Valine	0.03±0.00	0.02±0.00	0.70±0.07
Phenylalanine	-	-	0.14±0.06
Arginine	-	-	1.59±0.30
Lysine	-	-	-
Hydroxyproline	-	-	0.03±0.01
Histidine	-	-	0.02±0.00
Tyrosine	-	-	-
Tryptophan	-	-	1.08±0.17
Serine	0.02±0.00	0.02±0.00	0.19±0.02
Aspartic acid	0.01±0.00	0.01±0.00	0.07±0.02
Threonine	0.02±0.00	0.02±0.00	0.06±0.03
Methionine	-	-	0.04±0.04
Cysteine	-	-	-
Total	0.26±0.03	0.25±0.02	14.23±0.09

(-) = Not detectable

In order to study the oxidative and hydrolytic stabilities of marine PL emulsions without the interference from non-enzymatic browning reactions or factors such as the content of TAG, antioxidant and other residues that might be present in the marine PL, marine PL were further purified through acetone precipitation (Paper IV). Therefore, these marine PL are termed as ‘purified marine phospholipids’ or ‘AP’ in the present Ph.D. thesis. Acetone precipitation of marine PL was done according to the method described by Mozuraityte and co-workers (2008) and Schneider and Løvaas (2009) with some modifications. Due to the removal of TAG in purified marine PL, dispersions containing mainly liposomes were obtained through pre-emulsification and homogenization. Five liposomal dispersions were prepared with different levels of purified marine PL (AP) as shown in Table 5.5. A small amount of α -tocopherol was added to one of the marine PL dispersions to test the hypothesis that α -tocopherol is an efficient antioxidant to maintain the high oxidative stability of marine PL as proposed by several studies. The chemical composition of MPW before and after acetone purification is shown in Table 5.6.

Table 5.5: Experimental design for purified marine phospholipids (AP) dispersions

Formulations/ dispersions	Added tocopherol (mg/g of PL)	Phospholipids (%)	Total lipids (%)	Acetate- imidazole buffer (%)
APT	0.25	2.0	2.0	98
AP1	0.0	2.0	2.0	98
AP2	0.0	4.0	4.0	96
AP3	0.0	6.0	6.0	94
AP4	0.0	8.0	8.0	92

Table 5.6: Composition of MPW and AP (purified marine phospholipids).

Name	MPW	AP
Sources	Sprat fish meal	MPW after acetone precipitation
Total phospholipids (%)	41.50	66.23
Phosphatidylcholine PC (%)	18.30	21.34
Phosphatidylethanolamine PE (%)	4.70	9.21
Phosphatidylinositol PI (%)	2.10	2.76
Sphingomyelin SPM (%)	-	-
Lysophosphatidylcholine LPC (%)	3.40	11.15
Other phospholipids	8.90	23.12
Triglycerides (TAG)	40.0	-
Cholesterol (CHO)	2.0	ND
Free fatty acids	16.0	11.0
Peroxide Value (meq/kg)	0.81±0.04	1.66±0.21
Initial n-3 derived volatiles (mg/kg)	64.2	75.6
Strecker volatiles		
3-methylbutanal (mg/kg)	0.36±0.07	0.12±0.03
α-Tocopherol (mg/kg)	73.4	-
Induction period, IP (minutes)	1569±23	41±6
<i>After addition of α-tocopherol (600 mg/kg)</i>		<i>IP was not attained even after 6 days incubation</i>
ND= Not determined, (-) = Not detectable		

5.2.3 Part 3: Evaluation of non-enzymatic browning reactions in marine PL (paper III & V)

As mentioned earlier, non-enzymatic browning reactions were also investigated as a part of the study reported in paper III. This first pilot study gave a brief overview of non-enzymatic browning reactions in marine PL (paper III). In order to have a more comprehensive understanding of non-enzymatic browning reactions in marine PL emulsions, a model study was carried out. We hypothesized that non-enzymatic browning reactions could occur in marine PL emulsions through the interaction between lipid oxidation products with primary amine groups from PE and residues of amino acids that are present in marine PL. Therefore, liposomal dispersions were prepared from purified marine PL (LC and MPW), pure PC and PE authentic standards with and without addition of amino acids (as shown in Table 5.7). The purpose of adding amino acids to the selected dispersions was to investigate if the presence of amino acids or the participation of amino acids in non-enzymatic browning reactions would affect the oxidative stability of purified marine PL dispersions. Liposomal dispersions were incubated at 60 °C for 0, 2, 4 and 6 days. Both lipid oxidation and non-enzymatic browning reactions products in liposomal dispersions were measured.

PC and PE authentic standards were chosen for comparison as PC is the most dominant PL in the purified marine PL. In contrast, PE is the PL that usually involve in pyrrolisation. Furthermore, a molecular species comprising a palmitic acid (PA) at *sn-1* position and a docosahexaenoic acid (DHA) at *sn-2* position of PL was chosen for both PC and PE. This molecular species is one of the most dominant molecular species in marine PL (Le Grandois et al., 2009). On the other hand, lysine, leucine and methionine were chosen as the source of amine as they produced the most abundant Strecker degradation (SD) products in marine PL emulsions as determined in paper III. The details of this model study can be found in paper V. Different from the other studies (paper II, III, IV and VI), liposomal dispersions were prepared through sonication method at low power in this model study. In addition, two selected marine PL preparations were purified through Solid Phase Extraction (SPE) by using Sep-pak column containing aminopropyl modified silica.

Table 5.7: Experimental design for PL liposomal dispersions used in paper IV

*Liposomal Dispersions	Added amino acids (mg)			Concentration of amino acids (mg/mL)
	Lysine	Leucine	Methonine	
DPC	-	-	-	
DPCA	100	100	100	1.33
DPE	-	-	-	
DPEA	100	100	100	1.33
DLC	-	-	-	
DLCA	100	100	100	1.33
DMPW	-	-	-	
DMPWA	100	100	100	1.33

* *DPE & DPC are dispersions prepared from authentic standards phosphatidylcholine and phosphatidylethanolamine; DLC & DMPW are dispersions prepared from purified marine PL (LC & MPW); DPEA, DPCA, DLCA and DMPWA are dispersions added with amino acids, namely leucine, methonine and lysine.*

5.2.4 Part 4: Evaluation of marine PL fortified foods (paper VI).

As mentioned earlier, the ultimate goal of the present Ph.D. study was to explore the possibilities of using marine PL for food fortification. After investigating the physico-chemical properties (part 1) and oxidative stability (part 2) of marine PL emulsions, the obtained results led to a decision to carry out a pilot study on food fortification (part 4). Therefore, the main objective of this part was to investigate the effect of marine PL incorporation toward oxidative stability and sensory quality of fortified foods. Two marine PL preparations (LC and MPL), which gave a high oxidative stability were supposed to be used for food fortification, but they were not chosen due to several reasons. LC was not suitable for food fortification mainly due to its strong unpleasant odor, whereas the quality of MPL need to be improved prior to its use for food fortification. MPL was less oxidatively stable and had a higher degree of brownness than LC. Therefore, another two marine PL preparations were obtained for food fortification, namely krill phospholipids (MGK) of food grade quality and marine PL with an improved quality (MPN) from Triple Nine. The purpose of using marine PL preparations from different sources was to test the hypothesis that quality of fortified foods varies depending on the quality and source of marine PL used. The details of marine PL preparations used for food fortification can be found in Table 5.1.

A fermented milk system was used for marine PL incorporation due to several reasons. It is speculated that fermented milk system might provide a high oxidative stability for marine lipids (both fish oil and marine PL). The high viscosity in fermented milk product might decrease the diffusion of oxygen and pro-oxidants. In addition, the fermentation in the fermented milk system could lower the oxygen content and produce antioxidative compounds such as casein peptides and amino acids that might help to reduce lipid oxidation. Fortification of fermented milk product was made at 1 % marine PL incorporation. By judging the content of EPA and DHA in MGK, incorporation of 1 % MGK into fermented milk product will provide 110 mg EPA per 100 g fermented milk product and 70 mg DHA per 100 g fermented milk product.

Marine PL were used either in the neat form or in the pre-emulsified form for food fortification. The use of stabilized pre-emulsified marine PL is expected to provide a better oxidative stability. Therefore, marine PL emulsions were prepared at 2 different total lipid contents, 10 % and 50 % with the purpose to investigate the effect of lipid concentration and viscosity toward lipid oxidation (Table 5.8). Due to the issue of sensory acceptability, a low level of marine PL (0.5 % marine PL in combination with 9.5 % fish oil) was chosen for emulsion preparation prior to the food fortification. Similar to the study of oxidative stability in part 2, marine PL emulsions were stored at 2 °C for 32 days and oxidative stability of the emulsions were investigated through the measurements of PV and secondary volatiles. Furthermore, in order to confirm the hypothesis that marine lipid in PL form was more oxidatively stable as compared to fish oil in TAG form, the fermented milk product fortified with neat fish oil was used as comparison. The experimental design of marine PL fortified products is shown in Table 5.9. Food fortification with marine lipids was done by using Stephan mixer, where fermented milk product was mixed with marine lipids (either in the neat or the pre-emulsified form) under cold and vacuum condition. The fortified products were stored for 4 weeks (shelf life for commercial fermented milk product) at 5 °C. For more details of this part of experiment, refer to paper V.

Table 5.8 Experimental design of marine PL emulsions (used in paper VI)

Emulsion formulations	Marine phospholipids (%)		Fish oil (%)	Buffer acetate-imidazole *(%)
	MGK	MPN		
10 % MGK	0.5	-	9.5	90.0
50% MGK	2.5	-	47.5	50.0
10% MPN	-	0.5	9.5	90.0
50% MPN	-	2.5	47.5	50.0

**For food fortification, marine PL emulsions were prepared by using water instead of using buffer.*

Table 5.9 Experimental design of food fortification (used in paper VI)

Formulations	Sources of marine lipids used for fortification (g/100g)					
	Marine phospholipids (MGK)			Marine phospholipids (MPN)		Fish oil
	Neat	10 % emulsion	50 % emulsion	MPN	10 % emulsion	
Plain	-	-	-	-	-	-
Neat fish oil	-	-	-	-	-	1.0
Neat MGK	1.0	-	-	-	-	-
Neat MPN	-	-	-	1.0	-	-
10 % MGK	-	10.0	-	-	-	-
50 % MGK	-	-	2.0	-	-	-
10 % MPN	-	-	-	-	10.0	-
50 % MPN	-	-	-	-	-	2.0

5.3 Methodology

5.3.1 Characterisation of marine PL (paper II-IV)

Chemical compositions of marine PL were determined prior to the emulsions preparation. This includes the determinations of a) antioxidant content such as ethoxyquin, astaxanthin and tocopherol, b) fatty acid and phospholipids composition or lipid classes, c) iron content, d) peroxide value (PV) and free fatty acids (FFA), e) pyrrole content, f) amino acids composition and g) induction period by accelerated oxidation stability measurement using the Oxypress equipment.

5.3.2 Physico-chemical properties of marine PL emulsions (paper II)

Physical stability of marine PL emulsions was examined through the determinations of a) particle size distribution, b) zeta potential, c) microscopic examination and d) emulsion separation.

5.3.3 Hydrolytic and oxidative stability of marine PL (paper II-V)

Hydrolysis of PL in marine PL emulsions or dispersions was examined through the measurements of free fatty acids and PL content by ³¹P NMR, whereas the lipid oxidation was examined through the measurements of a) PV, b) tocopherol content, c) secondary volatiles by headspace analysis using solid phase microextraction (SPME) GC-MS or headspace analysis using dynamic headspace (DHS) GC-MS. Initially, only SPME was used to extract secondary volatiles from marine PL emulsions as it is a fast and simple method. However, fibre saturation was encountered when using SPME in some samples and therefore DHS was used to repeat the analysis. More details of comparison between these two methods can be found in paper VII.

5.3.4 Non-enzymatic browning reactions in marine PL (paper III-V)

Non-enzymatic browning reactions were determined through the measurements of a) SD products, b) pyrrole content, c) color changes, namely lightness and yellowness index (YI)

5.3.5 Sensory evaluation (paper VI)

Trained panellists were recruited to evaluate the marine PL fortified products using objective descriptive sensory profiling. Panellists had undergone three sessions of training and they agreed on the following attributes: fishy, rancid and sour both for aroma (orthonasal) and for flavour (retronasal). All sensory attributes were rated on an unstructured 15 cm line scale with anchor points 1.5 cm from each end. The data were recorded on computers by using the FIZZ program (Biosystems, Couternon, France). The obtained sensory data were calculated by determining the overall mean scores for intensity.

5.3.6 Statistical analysis (paper II - VI)

One way or two way ANOVA analysis followed by Tukey multiple comparison test (using a statistical package program Minitab 16) or *Bonferroni* multiple comparison test (using a statistical package program Graphpad Prism 4) were employed to evaluate the significant differences among the samples or the during storage. Significant differences were accepted at ($p < 0.05$). In some cases, multivariate analysis was performed by the Unscrambler (Unscrambler X, version 10.2) or LatentiX 2.0 (Latent5 Aps). The main variances in the data set were studied using principal component analysis (PCA). All data were centred and auto-scaled ($1/SD$) to equal variance prior to PCA analysis.

CHAPTER 6 SUMMARY OF RESULTS AND DISCUSSION

In this chapter, a brief discussion of experimental findings is presented. This includes discussion on different aspects of marine PL emulsions; physical and hydrolytic stabilities (part 1), oxidative stability (part 2) and non-enzymatic browning reactions (part 3). The last section of this chapter relates to the potential use of marine PL for food fortification (part 4). Further details relating to these experimental findings can be found in the relevant papers in the appendix.

6.1 Part 1: Physico-chemical properties of marine PL emulsions (paper II)

Marine PL are potential natural surfactants to prepare emulsions. They contain a high level of PC, which has amphiphilic properties. Therefore, the emulsifying property of marine PL or physico-chemical properties of marine PL emulsions was investigated (according to the experimental design shown in Table 5.2). Physico-chemical properties of marine PL emulsions are discussed in terms of emulsion separation (ES), hydrolytic stability, particle size distribution (PSD), zeta potential and microscopy inspection. Further details of the above mentioned work can be found in paper II.

6.1.1 A summary of physico-chemical properties of marine PL emulsions

As far as emulsion separation (ES) was concerned, emulsions prepared from a mixture of fish oil and marine PL had a tendency to cream or sediment, particularly when only 0.5 % marine PL was used in combination with 9.5 % fish oil. These emulsions also showed phase separation into four or three layers when stored at room temperature or 2 °C, respectively. In contrast, emulsions prepared from a higher percentage of marine PL (i.e. 3%) in combination with lower levels of fish oil (i.e. 7%) showed less creaming over time. Among the marine PL preparations (MPT, MPL and LC) used, the highest degree of ES was observed in the emulsions prepared from MPT (paper II). This phenomenon was most likely due to the lower level of PL, hydrolytic products (FFA and LysoPC) and the higher level of TAG in MPT as compared to MPL and LC (Table 5.1). Hydrolytic products were found in marine PL emulsions even before storage and these products originated from the marine PL preparation used as shown in Table 5.1. In addition, no PL hydrolysis was observed in marine PL emulsions during 32 days storage. According to Gritt and co-workers (1993), PL hydrolysis

is catalyzed by hydroxyl and hydrogen ions, and therefore PL hydrolysis was minimal at pH values near 6.5 to 7.

Creaming did not occur in emulsions prepared solely from marine PL, irrespective of the PL concentration investigated. As shown in Figure 6.1a, emulsions prepared solely from marine PL (MPT, MPL and LC) showed a monomodal particle size distribution (PSD) with a peak particle size around 0.10 μ m, which may indicate the presence of liposomes (Mozafari *et al.*, 2008). The presence of liposomes was confirmed by microscopy, seen in emulsion as bright orange tiny spots or tiny particles depending on the type of microscopy used (paper II). In addition to liposomes, larger droplets found in these emulsions most likely indicate the presence of a few oil droplets surrounded by PL monolayers (paper II). In addition, micelles with an average diameter of around 4 nm could also be formed from a monolayer of PL molecules with the hydrophobic fatty acid chains oriented towards the center of the micelle (Thompson *et al.*, 2006). However, measurement of micelles was impossible in the present study.

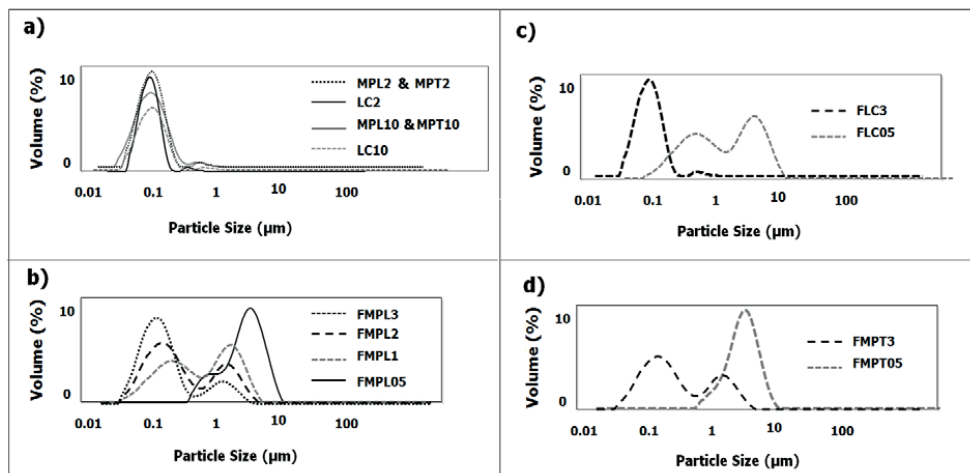


Figure 6.1: Particle size distribution of a) emulsions containing marine PL as the only lipid source, b-d) emulsions containing mixtures of fish oil and marine PL in different ratios after 32 days storage at 2°C. Value are the mean \pm standard deviation ($n=3$). Data are taken from paper II.

In contrast, the PSD of emulsions prepared from a mixture of fish oil and marine PL showed a bimodal PSD (Figure 6.1, b – d). In the bimodal PSD, emulsions prepared from 3 % of marine PL (FMPL3, FLC3 and FMPT3) had a larger population of smaller droplets and a smaller population of larger droplets. The opposite was observed for emulsions prepared from 0.5% of marine PL (FMPL05 & FLC05) (Figure 6.1 b & c). Smaller droplets (0.1 μm mean diameter) might indicate the presence of PL liposomes whereas larger droplets (2 μm mean diameter) might indicate the presence of TAG oil droplets surrounded by PL. Interestingly, a bimodal PSD was not obtained when MPT was used to prepare fish oil emulsions with 0.5 % of marine PL as exemplified by FMPT05 (Figure 6.1d). This could be attributed to the lower content of PL in MPT to form liposomes as compared to MPL and LC (Table 5.1).

6.1.2 Discussion of physical stability of marine PL emulsions

The physical stability of marine PL emulsions is discussed for two different groups; emulsions prepared solely from marine PL and emulsions prepared from a mixture of fish oil and marine PL. For emulsions prepared solely from marine PL, a high physical stability was obtained for all emulsions regardless of the percentage of marine PL used. The high physical stability in these emulsions was most likely due to: a) the presence of liposomes and micelles as they by nature are thermodynamically stable structures, b) the negative charge of the monolayer PL on the surface of the droplets which contributes to electrostatic stabilisation, and c) the presence of hydrolytic products such as FFA and lysoPL, which most likely contributes charge in addition to that of the PL themselves (Herman & Groves, 1992; Buszello et al., 2000). FFAs increased the negative surface charge of the droplets through their partitioning into the lipid layer at the o/w interface.

For emulsions prepared from a mixture of fish oil and marine PL, the physical stability of these emulsions decreased with an increase of fish oil or TAG level. The findings from the present Ph.D. study showed that emulsions prepared from a low level of marine PL (0.5 %) or a high level of fish oil (9.5 % fish oil) were found to be least physically stable. In order to maintain the high physical stability of these emulsions, at least 3 % of marine PL is required to cover the fish oil droplets completely and to avoid creaming and phase separation. Therefore, marine PL could be used as emulsifier to prepare physically stable emulsions and this finding confirmed the proposed hypothesis. In addition, this finding is in agreement with

the finding of Asai (2003), who also reported that phase separation was observed in o/w emulsion prepared from soybean oil and PC when PC content was not sufficient to cover oil droplets. Asai (2003) also reported that the coexistence of PL monolayer-encased oil droplets and liposomes is crucial to stabilize the o/w emulsion produced with PL as the only emulsifier. In general, the physical stability of both groups of emulsions can be improved if the marine PL used for emulsion preparation comprises a high level of phospholipids (especially PC), cholesterol, FFA and lysoPC or a low level of TAG. As mentioned earlier, the high level of PL could increase the formation of liposomes or PL monolayer to cover the TAG oil droplets, whereas the high level of hydrolytic products could increase the electrostatic stabilization. In addition, the presence of cholesterol could improve the physical stability of emulsion by increasing the rigidity of PL liposomes and their resistance toward degradation (Gritt et al., 1993). To summarize, the physical stability of marine PL emulsions was influenced by the chemical composition of marine PL used and this finding confirmed the proposed hypothesis.

6.2 Part 2: Oxidative stability of marine PL emulsions (paper III & IV)

Oxidative stability of marine PL emulsions could be influenced by the formulations or chemical compositions of marine PL used for emulsion preparation. This includes the contents of antioxidants and other minor residues that are present in marine PL. Therefore, the issue of oxidative stability was investigated and discussed from two aspects; a) emulsions prepared from untreated marine PL as reported in paper III (commercial marine PL were used for emulsion preparation without further treatment or purification), b) dispersions prepared from the purified marine PL as reported in paper IV (marine PL were purified through acetone precipitation prior to the dispersion preparation). The hypothesis of α -tocopherol being an efficient antioxidant to maintain the high oxidative stability of marine PL was also investigated in this part. For more details, refer to paper III and IV.

6.2.1 A summary of oxidative stability of marine PL emulsions/dispersions

As showed in paper III, oxidative stability of emulsions prepared from three different untreated marine PL (as shown by experimental design in Table 5.3) was further investigated through the measurements of hydroperoxides (PV) and secondary volatile oxidation products. Among these three marine PL preparations, LC provided the best oxidative stability to the marine PL emulsions. In addition, emulsion containing only marine PL (LC10) was more oxidatively stable than its corresponding emulsion containing both fish oil and marine PL (FLC3). Thus, these findings supported the hypothesis that n-3 LC PUFA in the PL form is more oxidatively stable than n-3 LC PUFA in TAG form. In contrast, emulsions prepared from MPL and MPW were more oxidized than their corresponding emulsions prepared from a mixture of fish oil and marine PL (FMPW3 & FMPL3). This opposite observation did not support the above-mentioned hypothesis. The results indicated that factors such as quality and chemical composition of marine PL might influence the oxidative stability of emulsions prepared. The high oxidative stability in emulsions prepared from LC could be explained by its quality and chemical composition (Table 5.1). In addition, both MPW10 and MPL10 emulsions had a lower content of α -tocopherol and therefore they were less oxidatively stable than emulsions FMPW3 and FMPL3. This might be due to the lower content of α -tocopherol in marine PL preparations used for emulsions preparation, namely MPL and MPW as compared to fish oil. Furthermore, emulsions prepared from MPL were more oxidatively stable than emulsions prepared from MPW due to the additional antioxidant (ethoxyquin) in MPL.

In paper IV, the oxidative stability of dispersions prepared from purified marine PL (according to experimental design in Table 5.5) was investigated. In general, purification of marine PL increased the total PL content, removed TAG, α -tocopherol and reduced the free fatty acids content (Table 5.6). Marine PL dispersions prepared from a higher level of purified marine PL (AP3 & AP4) were less oxidized than dispersions prepared from a lower level of purified marine PL (AP1 & AP2). A lower level of volatile increment (as illustrated by (Z)-4-heptenal) was found in AP3 & AP4 than AP1 & AP2 as shown in Figure 6.2. This finding supported the findings of many studies that marine PL had a high oxidative stability (chapter 2, section 2.2). Furthermore, dispersion containing α -tocopherol (APT) was less oxidized than corresponding dispersion with the same level of PL but without addition of α -tocopherol (AP1). This finding was further confirmed through the measurement of induction

period for untreated or purified marine PL by accelerated oxidation stability measurement (Table 5.6). The untreated marine PL showed a moderate induction period due to the presence of natural antioxidant. Its induction period decreased drastically after purification, this phenomenon might be attributed to the removal of α -tocopherol. However, addition of α -tocopherol to purified marine PL significantly extended again its induction period.

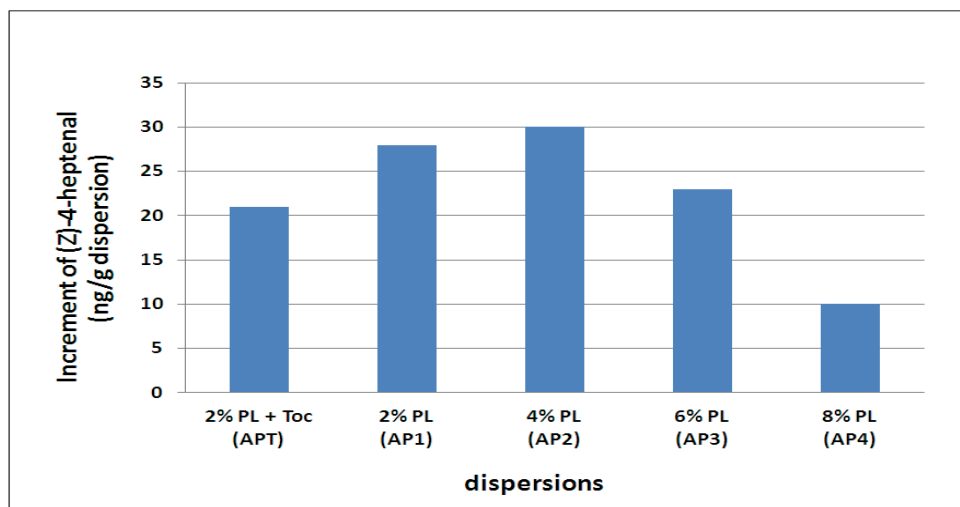


Figure 6.2: Increment of (Z)-4-heptenal in dispersions prepared from purified marine PL (AP) within 32 days storage at 2 °C. APT is a dispersion prepared from 2 % purified marine PL with 0.25 mg/g of α -tocopherol (Toc). AP1, AP2, AP3 and AP4 are dispersions prepared from 2 %, 4 %, 6 % and 8 % purified marine PL, respectively. Values are mean (n=3). Data are taken from paper IV.

6.2.2 Discussion of oxidative stability of marine PL emulsions/dispersions

As mentioned earlier in chapter 2 (section 2.2), many studies from the literature reported that marine PL were more oxidatively stable than fish oil despite the high degree of unsaturation (due to the high level of EPA and DHA) in marine PL (Nara et al., 1997; 1998; Cho et al., 2001; Moriya et al., 2007, Belhaj et al., 2010). As reviewed in paper I, several hypotheses were suggested to explain the high oxidative stability of marine PL as follows: a) their conformation of PUFA at the *sn*-2 position, b) synergistic effect of phospholipids on the antioxidant activity of α -tocopherol. However, more recent studies showed that c) the presence of pyrroles (antioxidative compounds produced in slightly oxidized PL through non-

enzymatic browning reactions) in marine PL might help to improve the oxidative stability of marine PL (chapter 4, section 4.2.4). This hypothesis was further confirmed by the findings from the present Ph.D. study (paper III & V). Even though marine PL were shown to have a high oxidative stability, their stability was greatly influenced by the level of antioxidants (α -tocopherol and pyrroles), pro-oxidants (transition metals and initial hydroperoxides) and other impurities (residues of amino acids) as observed in paper III and IV. For instance, emulsions prepared from marine PL preparation, namely LC with a low level of TAG and pro-oxidants, but a high level of α -tocopherol, PC and cholesterol were found to have high degree of oxidative stability (paper III). However, it cannot be ruled out that the low volatile oxidation products in emulsions prepared from LC was partly due to the high free amino acids content in LC (Table 5.4), which might participate in non-enzymatic browning reactions in marine PL. The effects of residues amino acids and non-enzymatic browning reactions toward lipid oxidation in marine PL are discussed in section 6.3.

As far as the antioxidant was concerned, a high oxidative stability was obtained for emulsion prepared from marine PL preparation containing a high level of α -tocopherol (paper III). The same observation was obtained for dispersion prepared from purified marine PL with addition of α -tocopherol (paper IV). This indicated that α -tocopherol is an efficient antioxidant to maintain the high oxidative stability of marine PL (paper III & IV). In addition, the presence of α -tocopherol could also influence the antioxidative properties of pyrroles (products from non-enzymatic browning reactions) that are present in marine PL as reported in paper III. This finding is in agreement with that of Hidalgo and co-workers (2007).

In addition, a high level of PL in marine PL preparation could produce emulsion of better oxidative stability due to its formation of larger population of liposomes from marine PL (paper II & III). Marine PC liposomes were shown to have a tighter molecular conformation, which might decrease the attack of free radicals and oxygen toward PUFA in the bilayers of the liposomes (Nara et al., 1997; 1998). For instance, emulsion FLC3 was shown to contain a higher level of liposomes than emulsions FMPL3 (the presence of liposomes in marine PL emulsions was confirmed by the measurement of PSD and microscopy inspection as reported in paper II). Therefore, the presence of liposomes might be one of the reasons that FLC3 was more oxidatively stable than FMPL3 (paper III).

The other reason is the presence of a high level of cholesterol in LC, which could improve both the physical and oxidative stabilities of emulsions prepared (paper II & III). As also suggested by several other studies (Nara et al., 1998, Monroig et al., 2003), the addition

of cholesterol improved the oxidative stability of liposome dispersions prepared from marine PC. Cholesterol has a condensing effect on the PC liposome (Finean, 1990). It could increase the rigidity of 'fluid state' liposomal bilayers and thus improve the oxidative stability of liposomes (Fiorentini et al., 1989).

In addition, a high level of pro-oxidants such as transition metals and initial hydroperoxides in marine PL preparations could decrease the oxidative stability of emulsions prepared. As shown in paper III, emulsions prepared from marine PL preparations, namely MPL and MPW were less oxidatively stable than that of LC and this phenomenon might be attributed to the higher level of pro-oxidants in both MPL and MPW (Table 5.1). According to Mozuraityte and co-workers (2006a), the oxidative stability of liposome dispersions prepared from cod phospholipids decreased after addition of transition metals. The presence of transition metals, Fe^{2+} and Fe^{3+} could promote lipid oxidation by decomposing lipid hydroperoxide into free radical. In addition, the high level of iron could also induce lipid oxidation through the fast fixation of positively charged iron to negatively charged PL liposomes that are present in the emulsion (Mancuso et al., 1999). Several studies (Mei et al., 1998a; 1998b; Minotti & Aust, 1989) reported that the interaction between lipid hydroperoxides and transition metals is the main cause of lipid oxidation. In conclusion, the finding from the present Ph.D. study showed that the oxidative stability of marine PL emulsions/dispersions was influenced by the quality, chemical composition and source of marine PL used and this finding confirmed the proposed hypothesis.

6.3 Part 3: Non-enzymatic browning reactions in marine PL (paper III & V)

Secondary oxidation products in marine PL especially the unsaturated and polyunsaturated aldehydes are very reactive toward the primary amine groups of amino phospholipids or amino acids/protein. Therefore, their presence could lead to the formation of highly colored pyrrole polymers and cause non-enzymatic browning reactions in marine PL (refer to chapter 4, section 4.1). In general, oxidation products of lipids contribute to non-enzymatic browning through formation of colored pyrrole polymers and Strecker degradation (SD) of amino acids. In order to obtain a better understanding of non-enzymatic browning reactions in marine PL, these reactions were investigated in untreated marine PL emulsions (paper III) and marine PL liposomal system comprising primary amine groups from PE and amino acids (paper V).

6.3.1 A summary of non-enzymatic browning reactions in untreated marine PL emulsions.

Non-enzymatic browning reactions (SD and pyrrolisation) were investigated in emulsions prepared from untreated marine PL (according to experimental design in Table 5.3). At least 8 different types of SD products were found in emulsions prepared from marine PL preparations (MPL, MPW and LC) through SPME GC-MS/DHS GC-MS determination (paper III). To the best of our knowledge, this is the first study reports the generation of SD products in marine PL emulsions. 3-methylbutanal, dimethyldisulphide and 2-methyl-2-pentenal were the most dominant SD products degraded from leucine, methionine and lysine in marine PL emulsions. The hypothesis that SD products degraded from amino acids was further confirmed by the analysis of amino acids composition in marine PL preparations. A high level of SD products was found in LC emulsion and this could be attributed to the high level of amino acid residues in LC (Table 5.4). In contrast, a low level of SD products was found in emulsions prepared from MPW and MPL, which contained a low level of amino acids (Table 5.4). Among the measured SD products, two of them slightly increased in emulsions prepared from MPW after 32 days storage. Therefore, SD might occur at low reaction rate in marine PL emulsions during their storage at low temperature (2 °C). However, most of the SD reaction seemed to occur in marine PL during their manufacturing process. In addition to SD products, two types of pyrroles (hydrophobic and hydrophilic) were found in marine PL emulsions as shown in Figure 6.3.

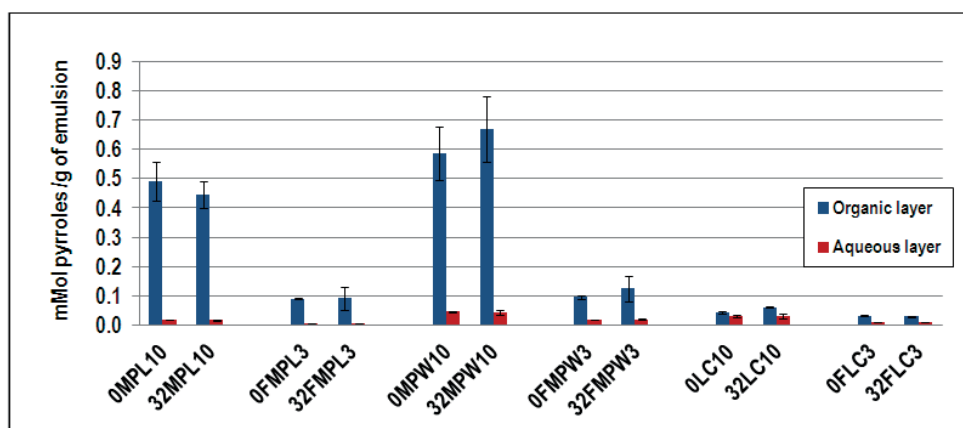


Figure 6.3: Comparison of hydrophobic pyrroles (organic layer) and hydrophilic pyrroles (aqueous layer) in marine PL emulsion before (0) and after (32) days storage at 2°C. Values are mean±standard deviation (n=2). Data are taken from paper III.

Pyrroles are responsible for browning development in marine PL. Therefore, color changes (as illustrated by lightness, L* and yellowness index, YI) in marine PL emulsions during storage were measured as the indication of pyrrolisation. For more details of color changes in marine PL emulsion, refer to paper III. The main findings of pyrrolisation in marine PL emulsions are summarized as follows: a) most of the pyrrolisation occurred in marine PL during their manufacturing processes and the level of pyrroles in marine PL emulsions did not seem to change significantly during 32 day storage, b) the level of hydrophobic pyrroles was higher than hydrophilic pyrroles in all emulsions, c) the level of hydrophobic pyrroles in emulsions was ranked as follows: MPW > MPL > LC (according to the marine PL preparations used).

6.3.2 A summary of non-enzymatic browning reactions in purified marine PL dispersions.

A model study was carried out to further investigate the non-enzymatic browning reactions in marine PL and to confirm the proposed mechanisms in section 6.3.3. Liposomal dispersions were prepared from pure PC, PE compounds and purified marine PL according to experimental design as shown in Table 5.7. The main findings drawn from this model study are summarized as follows: a) SD products were only found in liposomal dispersions containing primary amine group either from PE or amino acids, b) PE pyrrolisation only occurred in liposomal dispersion containing PE, whereas amino acid pyrrolisation only occurred in liposomal dispersions containing amino acids. In addition, no pyrroles was found in PC dispersion, which contain no primary amine group, c) A higher degree of lipid oxidation and browning was observed in liposomal dispersions without amino acids than liposomal dispersions with amino acids added. The browning in PC liposomal dispersion was not due to the pyrrolisation as confirmed by the absence of pyrroles in PC dispersion. For more details, refer to paper V.

6.3.3 Proposed mechanisms for non-enzymatic browning reactions in marine PL.

Several mechanisms were proposed for non-enzymatic browning reactions in marine PL (Figure 6.4). It is speculated that extraction of marine PL at high temperature cause lipid oxidation and form firstly secondary volatile oxidation products and subsequently tertiary lipid oxidation products such as unsaturated epoxy keto fatty esters, epoxyalkenals and hydroxyalkenals. Tertiary lipid oxidation products are reactive toward primary amine group from PE and residues amino acids that are present in marine PL (Zamora et al., 2007). Lipid

oxidation of n-3 fatty acids amongst other produces 2, 4-heptadienal (secondary volatile oxidation products), which subsequently form 4, 5 (E)-epoxy-2-(E) heptenal with two oxygenated function groups (tertiary lipid oxidation products, these products could not be detected by SPME-GC/MS). Zamora and co-workers (2007) suggested that the presence of two oxygenated, namely one carbonyl group and one epoxy or hydroxyl group is required for the SD reaction to occur. An example of SD is shown by mechanism A (Figure 6.4), this reaction could occur between an epoxyalkenal (4, 5 (E)-epoxy-2-(E) heptenal) and an amino acids (leucine) producing 3-methylbutanal and a hydroxyl amino compound, which could be further degraded to form 2-methylpyridine. In addition, secondary lipid oxidation products such as alkadienals and ketodienes could degrade amino acids to their corresponding SD products when secondary lipid volatiles are further oxidized under appropriate conditions (Zamora et al., 2007).

Pyrrolisation could occur between tertiary oxidation products of lipid with primary amine group from phosphatidylethanolamine (PE) or amino acids/protein residues that are present in marine PL. As shown in Figure 6.4 (mechanism B and C), if a reaction takes place between tertiary lipid oxidation products with primary amine group present in PE, the pyrroles produced are most likely to be hydrophobic, but if a reaction takes place with amino group of amino acids or protein, the pyrroles produced are most likely to be hydrophilic. This hypothesis was further confirmed by the findings in paper V as mentioned earlier. Between PE and amino acids, the amino group of PE undergoes pyrrolization 10 times more readily than the amino group of amino acids. This is due to the close proximity of the generation place of lipid oxidation products to the amino group of PE (Zamora et al., 2005). The obtained results in the present Ph.D. study confirmed the hypothesis that more hydrophobic pyrroles were formed than hydrophilic pyrroles in marine PL (paper III). As mentioned in chapter 4 (section 4.2.2), two types of pyrroles could be produced during the pyrrolization process, namely N-substituted pyrroles which are stable and 2-(1-hydroxyalkyl)pyrroles, which are unstable. 2-(1-hydroxyalkyl)pyrroles could further polymerize to form pyrroles in dimer or polymer form with different antioxidative properties as reported by Hidalgo and co-workers (2003). Slightly oxidized PE could produce pyrroles in dimer form, which has better antioxidative properties than pyrroles in the polymer form as polymerization could decrease the antioxidative property of pyrroles (Hidalgo et al., 2003). In fact, pyrroles formation and polymerization are responsible for the browning development in the systems containing both tertiary lipid oxidation products/carbonyl derivatives and primary amine group (Zamora et al

2000; 2004). The hypotheses relating to pyrroles formation in marine PL and their antioxidative property were further confirmed by the findings in paper V.

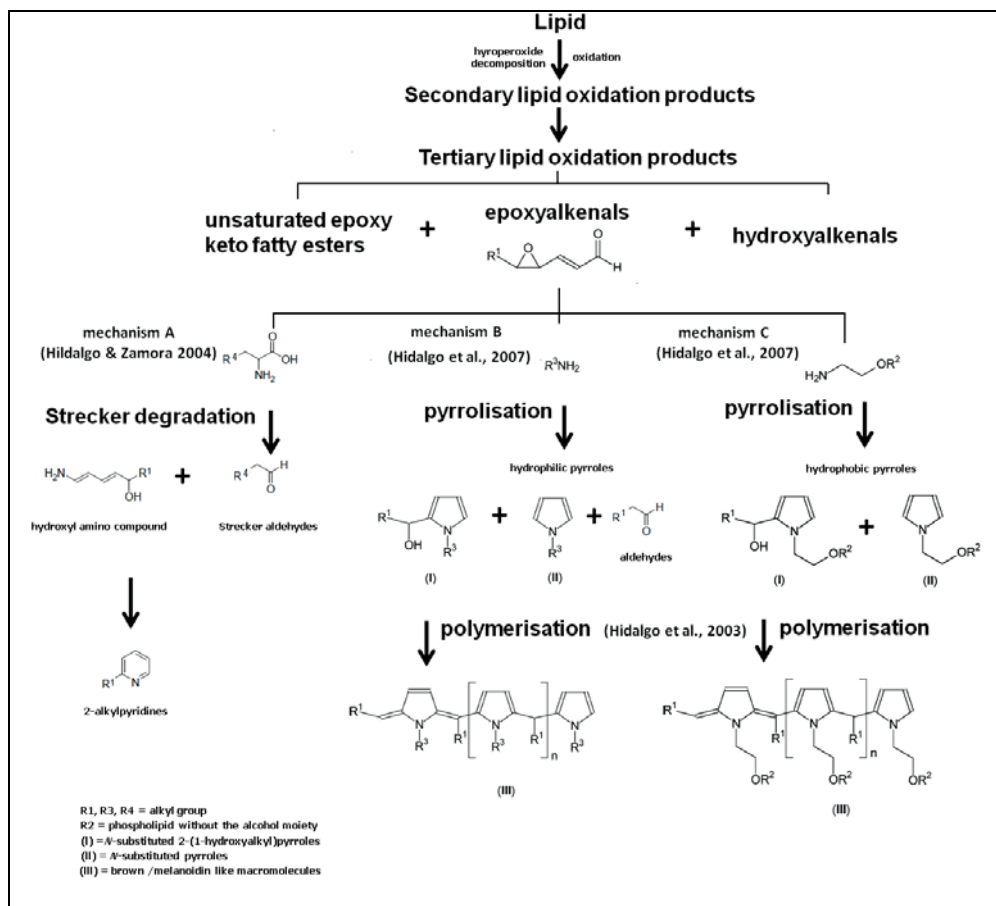


Figure 6.4: Proposed mechanisms for non-enzymatic browning reactions in marine PL

6.3.4 Discussion of lipid oxidation and non-enzymatic browning in marine PL

In this section, a discussion of lipid oxidation and non-enzymatic browning is made for marine PL based on the findings from paper III and model study (paper V). As mentioned earlier in chapter 5, non-enzymatic browning reaction was investigated only in emulsions prepared from LC, MPW and MPL or liposomal dispersion prepared from purified LC and MPW, pure PC and PE authentic standards. The degree of non-enzymatic browning reactions (pyrrolisation or SD) in marine PL could be influenced by: a) the chemical composition of

marine PL such as the level of amino acids residues and PE, b) marine PL manufacturing processes such as temperatures and conditions of marine PL extraction. As mentioned in chapter 5, both MPW and MPL were extracted from fish meal at high temperature, whereas LC was extracted from fish by-product through enzymatic hydrolysis at low temperature. Therefore, different types and levels of both pyrroles and SD products were found in emulsions prepared from MPW, MPL and LC (paper III). The high level of pyrroles in both MPW and MPL might be attributed to the high temperature used in fish meal production prior to the extraction of marine PL from this fish meal. The use of high temperature in fish meal production could cause lipid oxidation and therefore pyrrolisation might occur in fish meal even before marine PL production. In addition, pyrrolisation in fish meal could be influenced by the quality of fish used for fish meal production. The condition, temperature and time used to store fish prior to their use to produce fish meal could influence the quality of both fish meal and marine PL produced. As also shown by the findings from model study (paper V), lipid oxidation increased and subsequently led to an increase of pyrroles formation as incubation progressed from 0 day to 6 days.

In contrast, degradation of amino acids was higher than pyrrolization in emulsions prepared from LC (paper III). This phenomenon might be attributed to the chemical composition of LC with a high level of free amino acids or its manufacturing process at low temperature. The finding from the model study also showed that SD was high in liposomal dispersions with amino acids added (paper V). Although the typical SD occurs at high temperature, SD seems to be high in LC marine PL preparation, which was produced at low temperature. It is undeniable that SD could also occur at low reaction rate in marine PL emulsions during at low temperature as reported in the present Ph.D. study (paper III). This finding is in agreement with several other studies, who reported that interaction between amino acids and lipid oxidation products could occur at low temperature such as 25 °C and 37 °C (Pripis-Nicolau et al., 2000; Hidalgo & Zamora 2004; Ventanas et al., 2007). In addition, the presence of pyrroles in LC marine PL preparation implies that pyrrolisation, most probably protein pyrrolisation could occur in marine PL production at low temperature as also suggested by Hidalgo and co-workers (1999). In general, both the chemical composition and marine PL manufacturing process seems to play an important role in determining the non-enzymatic browning reactions in marine PL emulsions.

Browning development in marine PL might be attributed to the formation of both pyrroles and oxypolymers (paper V). As shown in Table 5.1 and 5.4, marine PL contain PE,

residues amino acids and a high level of EPA and DHA. Therefore, the lipid oxidation products generated from EPA and DHA in marine PL might involve in oxypolymerisation and form brown oxypolymers. As also shown by the finding from model study (paper V), PC do not contain primary amine group and therefore PC might contribute to browning development through oxypolymerisation. In contrast, primary amine group from PE and residues amino acids might involve in pyrrolisation and form pyrroles. However, further investigation is required to find out which reaction (pyrrolisation or oxypolymerisation) contributes more to browning development in marine PL. Furthermore, the increase of lipid oxidation could increase both the SD and browning development in marine PL as also shown by the findings in paper V. Several studies also reported that lipid oxidation was positively correlated with non-enzymatic browning development in marine PL liposomes (Thanonkaew et al., 2006b; 2007).

As mentioned earlier, lipid oxidation firstly produces oxidation products that subsequently react with primary amine group to produce SD products or antioxidative compounds (pyrroles) through non-enzymatic browning reactions. Then, the produced antioxidative compounds might inhibit lipid oxidation in marine PL. Lipid oxidation and non-enzymatic browning reactions are closely linked in marine PL system as in other systems where both lipids and amine groups are present. For instance, the low level of secondary volatile oxidation products in emulsions prepared from LC was partly due to the interaction of lipid oxidation products with primary amine group from amino acids to form pyrroles (most probably pyrroles in dimer form) (paper III). This hypothesis was further confirmed by the finding from model study (paper V), which reported that a gradual decrease or disappearance of lipid oxidation products was found in PL liposomal dispersions containing amino acids as non-enzymatic browning reactions progressed.

In addition to the reasons mentioned in section 6.2.2, the other reasons for high oxidative stability in emulsions prepared from LC are stated as follows: a) the presence of pyrroles in dimer form, which were formed through non-enzymatic browning reactions. Pyrroles in dimer form were shown to have a better antioxidative property than pyrroles in polymer form, which were formed through polymerization of pyrrole in monomer form (Hidalgo et al., 2003), and b) a high level of free amino acids, which were shown to have antioxidative properties as confirmed by the finding from model study (paper V). In contrast, the lower oxidative stability of MPW and MPL than LC might be attributed to a) their pyrroles in polymer form, which were formed in the later stage of lipid oxidation and

therefore had low antioxidative properties, and b) the low level of free amino acids. In conclusion, both the chemical composition and products from non-enzymatic browning reactions in marine PL seemed to affect the oxidative stability of marine PL and these findings confirmed the proposed hypotheses.

6.4 Part 4: Food fortification with marine PL (paper VI)

The use of marine PL for food fortification is a new challenge in food industries. This is due to the presence of brown pigments such as pyrroles/oxypolymer (products of non-enzymatic browning reactions), dark red pigment (astaxanthin in krill PL) and unpleasant odor in most of the current marine PL that are available in the market. Even though marine PL are shown to have antioxidative properties, marine PL are still susceptible to lipid oxidation due to their high level of n-3 LC PUFA, namely EPA and DHA (chapter 2, section 2.2). Therefore, the different aspects of marine PL fortified foods such as the oxidative stability, sensory and physico-chemical properties need to be evaluated on product basis prior to the development of marine PL functional foods. In the first part of this section, the findings from the present Ph.D. study are summarized and discussed (section 6.4.1 and paper VI). In the second part, the potential use of marine PL for food fortification is briefly discussed based on the findings from the present Ph.D. study and compared with those from literature (section 6.4.2). Discussion is made based on the above-mentioned aspects with a special emphasis on oxidative stability of marine PL fortified foods.

6.4.1 A summary of findings for marine PL fortified food (fermented milk products)

Incorporation of marine PL either in the neat (1% marine PL) or in emulsion form (a mixture of fish oil, 0.95 % and marine PL, 0.05 %) significantly increased the lipid oxidation in fermented milk products. This observation was shown by the measurements of PV (Figure 6.5) and was further confirmed by the measurement of secondary volatile oxidation products in fortified products. In terms of neat marine lipids fortification (1 g of marine lipid per 100 g of fermented milk product), product fortified with neat MPN was more oxidized than product fortified with neat MGK and followed by neat fish oil. The same order of lipid oxidation was obtained for fortification in emulsion form (10 g of 10 % marine PL emulsion per 100 g of fermented milk product or 2 g of 50 % marine PL emulsion per 100 g fermented milk product). This phenomenon was most likely due to the different contents of α -tocopherol in fish oil and marine PL (MGK > Fish oil > MPN, refer to Table 5.1). In addition, the poorer quality of marine PL as compared to fish oil might also affect the lipid oxidation and

subsequently the quality of the fortified products. Both marine PL preparations (MPN & MGK) were found to contain impurities such as trace hydroperoxides, iron and residues amino acids. However, MGK has a better quality than MPN due to its lower content of iron and a higher level of PC and tocopherol. Therefore, the quality of fortified products was greatly influenced by the quality of marine lipids used for fortification and this finding confirmed the proposed hypothesis.

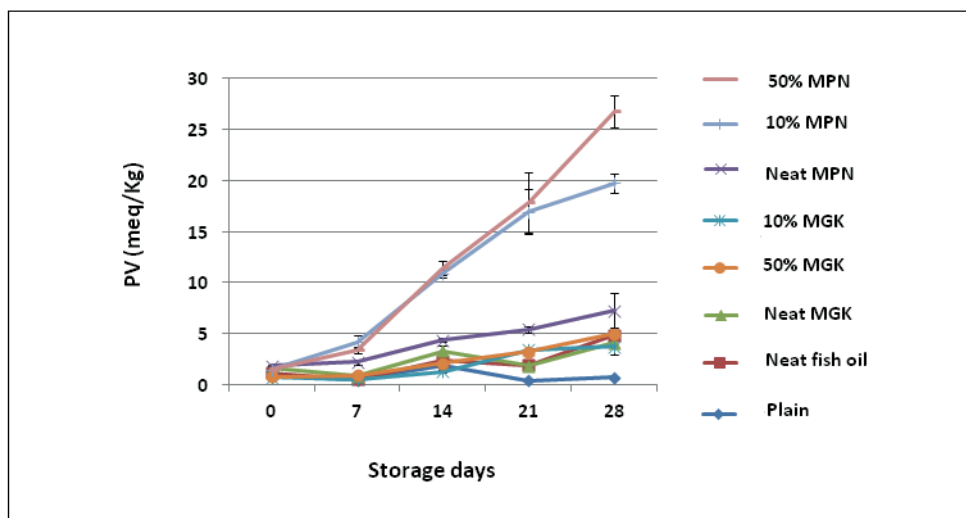


Figure 6.5: Changes of PV in plain and fortified products during 28 days storage at 5 °C. Values are means \pm standard deviation (n = 2).

Surprisingly, the rank order of marine PL oxidation in fermented milk system was different from that in the corresponding emulsion system (refer to paper VI). In addition, sensory evaluation was carried out for both plain and fortified products except the product fortified with neat marine PL. This is because the fishy and other unpleasant flavors were already pronounced in these fermented milk products even at the start of the experiment. Incorporation of marine lipids either fish oil or marine PL into fermented milk system did not affect the sourness of the fortified products, but increased the fishiness and rancidness of the fortified products. The obtained results from sensory evaluation is in agreement with the results from PV and secondary volatile oxidation products, that MPN fortified products were the most oxidized, followed by MGK fortified products and the neat fish oil fortified product was the most oxidative stable system. In summary, incorporation of marine PL into

fermented milk products decreased the oxidative stability and sensory quality of fortified products and this finding did not support the proposed hypothesis.

6.4.2 Discussion of findings and the potential use of marine PL for food fortification

As mentioned earlier, the findings from the present Ph.D. study showed that fortification of fermented milk product with a mixture of fish oil and marine PL did not provide a better oxidative stability than fortification with only fish oil (paper VI). This unexpected result is mainly due to the quality of current marine PL that are available in the market for food application. Incorporation of neat/pre-emulsified marine PL into fermented milk system increased lipid oxidation in fortified products. The finding is partially in agreement with the findings from other studies which also reported that foods fortified with neat marine PL from krill were susceptible to lipid oxidation (Kassis et al., 2010; 2011; Pietrowski et al., 2011; Sedoski et al., 2012). Pietrowski and co-workers (2011) developed surimi based seafood products fortified with n-3 PUFA rich oils from flaxseed, algae, menhaden, krill and a blend of these oils (flaxseed: algae: krill, 8: 1: 1). Fortification of surimi based seafood products with n-3 PUFA rich oils was carried out at 9 % (9 g oil per 100 g surimi paste). They reported that krill oil fortified surimi based seafood products were most oxidized due to the highest level of n-3 LC PUFA (EPA and DHA) in krill oil as compared to other n-3 PUFA rich oils. In addition, the same research group also developed novel nutraceutical egg products fortified with n-3 PUFA. The egg products were developed by using fresh egg white, freeze-dried egg white and egg yolk was substituted with the same n-3 PUFA rich oils as mentioned previously (with an incorporation level of 10 % neat oil).

Their studies reported the same finding that krill oil fortified nutraceutical egg products were most oxidized (Kassis et al., 2010; 2011). The two marine PL preparations used for fortification in the present Ph.D. study also comprised a higher level of EPA and DHA than fish oil (total content of EPA and DHA is presented in % area GC; 28.00 in MGK; 32.80 in MPN and 20.84 in fish oil, refer to Table 5.1). Therefore, the finding from the present Ph.D. study (paper VI) seemed to be in agreement with those from literature. However, the findings from part 1, 2 and 3 in the present Ph.D. study as well as other studies (Cho et al., 2001; Moriya et al., 2007, Belhaj et al., 2010) suggested that the high level of EPA and DHA in marine PL might not be the only reason for the high lipid oxidation in marine PL fortified products. Thus as mentioned earlier, marine PL were shown to have a better oxidative stability than fish oil despite the high degree of unsaturation in marine PL

(chapter 2, section 2.2). In addition, the findings from the present Ph.D. study showed that the oxidative stability of marine PL was influenced by the quality, chemical composition and sources of marine PL (paper III). Therefore, the different qualities, chemical compositions and source of marine PL used for fortification might be a more reasonable explanation for the different oxidative stability and sensory property of marine PL fortified foods (paper VI).

In addition, it was not possible to compare the oxidative stability of marine PL fortified foods in the present Ph.D. study directly with that of literature as different marine PL were used in different studies. The quality of krill oil (the level of impurities such as iron) used for fortification was not investigated in the studies of surimi based seafood and nutraceutical egg products. The finding from the present Ph.D. study showed that iron in marine PL played an important role in oxidation of fermented milk system (paper VI). Marine PL with different level of iron might behave differently in different food systems. In addition, the oxidative stability of marine PL in emulsion system was different from food systems due to the interaction between marine PL and other components in food system. Therefore, evaluation of quality of marine PL prior to their use for food fortification is important to provide a clear overview of oxidative stability of fortified foods.

In terms of sensory property, incorporation of marine PL emulsion increased both the fishiness and rancidness of fortified products as compared to control despite the low incorporation level of 0.05 % marine PL combined with 0.95 % fish oil (due to the addition of 1 g of 10 % marine PL emulsion prepared from a mixture of 0.5 % marine PL and 9.5 % fish oil, refer to paper VI). Although the incorporation of marine PL emulsion did not change the color and texture of the fermented milk products, the use of neat marine PL increased the yellowness/redness of the fortified products (data not shown). In contrast, surimi based seafood products fortified with neat krill oil still showed an acceptable sensory property despite the incorporation level is 9 %, which is much higher than the incorporation level used in the present Ph.D. study. In terms of physico-chemical properties, there were no changes in texture properties, but the color of of krill oil/blend oils fortified surimi based seafood products were darker than other fortified products (Pietrowski et al., 2011). The same color observation was obtained for novel nutraceutical egg products fortified with 10 % neat krill oil. Nutraceutical egg products with acceptable sensory and color properties were obtained when the krill oil incorporation level was reduced to 1 % (Kassis et al., 2011). Krill oil incorporation level at 1 % reduced the content of red pigment (astaxanthin) and thus its effect on color properties of fortified egg products.

Food fortification with marine PL requires expertise and skills as marine PL contain a high level of EPA and DHA, trace impurities and unpleasant odor, which may affect the quality of marine PL fortified foods. There are several precautions that food manufacturers must beware of in producing marine PL functional foods as stated as follows: a) marine PL incorporation level need to be evaluated on product basis as marine PL might behave differently in different food systems. For instance, incorporation level of krill oil at 9 % into surimi based seafood products did not adversely affect the sensory property of the fortified products, but this was not the case for fermented milk product despite the very low incorporation level of marine PL. It is easier for consumers to accept the fishy flavor in surimi based seafood products than in fermented milk system. Therefore, addition of other flavors/fruits such as strawberries is necessary to mask the fishy flavor in fermented milk system, b) the quality of current marine PL need to be improved or marine PL need to be refined prior to their use for food fortification, c) stabilization of marine PL in both emulsion and food systems with additional antioxidants or metal inactivators such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ascorbyl palmitate, ethylenediaminetetraacetic acid (EDTA), astaxanthin and α -tocopherol. Antioxidant such as α -tocopherol might be a good choice to improve the oxidative stability of marine PL fortified foods as it was proven to be an efficient antioxidant to maintain the high oxidative stability of marine PL (paper IV).

CHAPTER 7 CONCLUSION AND FUTURE PERSPECTIVES

The findings from the present Ph.D. study provided crucial information on the different aspects of marine PL emulsions and dispersions including the related physico-chemical properties, oxidative stability and non-enzymatic browning reactions. In addition, this study proposed several mechanisms for non-enzymatic browning reactions in marine PL and investigated the relationship between non-enzymatic browning reactions and lipid oxidation in marine PL system. Overall, the present Ph.D. study provided new insights into the oxidative stability of marine PL and knowledge on the quality of marine PL fortified products.

Marine PL could be used to prepare emulsions as n-3 LC PUFA delivery system without the addition of other emulsifiers. This is due to the high content of PC in marine PL, which has amphiphilic properties. Therefore, physically stable emulsions containing only marine PL could be prepared by using 2-10 % marine PL. In contrast, formulation of physically stable emulsions containing a mixture of marine PL and fish oil required at least 3% of marine PL to avoid creaming and phase separation. The high physical stability of marine PL emulsions was most likely due to the coexistence of micelles, liposomes and emulsified oil droplets. However, further studies are required to confirm this hypothesis. Such studies may include: a) measurement of liposomes diameter by using dynamic light scattering, b) determination of trapped aqueous volume of liposomes, c) estimation of monolayer-bilayer equilibrium of fish oil/PL mixtures by the measurement of spreading and collapse pressures. In general, the physical stability of marine PL emulsions varied depending on their formulations and chemical composition of marine PL used for their preparation.

In contrast to the findings of other studies, the oxidative stability of emulsions prepared from marine PL containing n-3 LC PUFA in PL form was not always higher than that of emulsions prepared from fish oil containing n-3 LC PUFA in TAG form. Other factors such as quality, source and chemical composition of marine PL also influenced the oxidative stability of marine PL emulsions. In general, marine PL emulsions showed high oxidative stability when they were prepared from marine PL of high quality with a low content of pro-oxidants (transition metals and initial hydroperoxides) and with a high content of α -tocopherol and PC. In addition, the presence of cholesterol and antioxidative compounds such as free amino acids and pyrroles (formed via non-enzymatic browning reactions) seemed to

improve the oxidative stability of marine PL emulsions. Although PL itself has high oxidative stability, its oxidative stability was greatly improved by the presence of α -tocopherol. α -tocopherol was proven to be an efficient antioxidant to maintain the high oxidative stability of marine PL. In addition, hydrolysis of PL in marine PL emulsions was minimal at pH 7. Based on these results, possible future studies could be carried out to improve the oxidative stability of marine PL emulsions by adding natural antioxidants such as rosemary extract, ascorbic acid and green tea extract or synthetic antioxidants such as BHT, TBHQ, EDTA, etc to the emulsions in addition to the α -tocopherol that is naturally present in marine PL.

It is speculated that non-enzymatic browning reactions including pyrrolisation and Strecker degradation (SD) mainly occur in marine PL during their manufacturing process due to the interactions between the products of lipid oxidation with the primary amine group from PE or amino acids/protein residues that are present in marine PL. The occurrence of SD was observed through the measurement of Strecker aldehydes and other degradation products from amino acid residues that were present in the marine PL. On the other hand, the occurrence of pyrrolisation was observed through the measurement of hydrophobic and hydrophilic pyrroles, which were formed via PE pyrrolisation and amino acid pyrrolisation, respectively. In addition, the result from the model study on liposomal dispersions showed that the browning development in marine PL was most likely due to PE and amino acid pyrrolisation or oxypolymerisation of lipid oxidation products in marine PL. The content of pyrroles, SD products and the degree of browning in marine PL might be influenced by chemical composition of marine PL and their manufacturing process. In the present Ph.D. study, an attempt has been taken to identify the molecular structure of pyrroles that were present in marine PL such as derivatization of non-volatile pyrroles to volatile pyrroles, followed by determination of pyrroles using GC-MS. However, the presence of low concentration of pyrroles and yet high content of PL or other lipid components in marine PL complicated the pyrroles identification process and therefore no promising data was obtained. In order to further confirm the proposed mechanisms of non-enzymatic browning reactions in marine PL, further studies are required such as a) isolation and purification pyrroles from marine PL prior to their determination by GC-MS; b) determination of oxypolymers in marine PL by high performance size exclusion chromatography and c) study of the possible formation of tertiary lipid oxidation products and their reaction with PE and amino acids in marine PL.

In the present Ph.D. study, several attempts were taken to investigate different aspects of marine PL, namely the physico-chemical properties and oxidative stability prior to their applications in food system. The incorporation of marine PL into fermented milk product adversely affected its oxidative stability and sensory quality even when low percentage of marine PL in combination with fish oil was used for fortification. This negative effect is due to the low quality of current marine PL preparations that are available in the market. Incorporation of marine PL regardless of their form (neat or pre-emulsified) decreased the oxidative stability and increased the fishiness and rancidness of the fortified product. In general, the oxidative stability and sensory quality of the marine PL fortified product varied depending on the quality and source of marine PL used. Although the quality of current marine PL in capsules form meets the Generally Recognized As Safe (GRAS) for dietary supplements (where marine PL are present in bulk oil system), the presence of trace amounts of iron and hydroperoxides in marine PL might have different impacts toward lipid oxidation in emulsified food systems. Therefore, it is necessary to use high quality marine PL (with a low content of transition metals, initial hydroperoxides and a high content of antioxidant) in order to obtain marine PL fortified products of satisfactory quality. Overall, the findings from the present Ph.D. study provided food industries and academia inspirations to improve the quality of current marine PL. Further studies could be carried out in the future to improve the marine PL manufacturing process such as the use of enzymatic hydrolysis or low temperature for marine PL extraction, new refining or deodorization method for marine PL to remove the brown color and other impurities that are present in marine PL, etc.

The use of marine PL for food applications is a new area in food industry. Due to the high content of n-3 LC PUFA in marine PL, marine PL fortified foods are still susceptible to lipid oxidation even when marine PL of high oxidative stability are used. Therefore, studies are required in the future to improve the oxidative stability of marine PL in real food systems. For instance, a) the use of appropriate level of marine PL for food fortification should be evaluated on product basis as marine PL behave differently in different food systems, b) the use of marine PL in liposome form instead of emulsion form as nutrient delivery system. The next frontier in marine PL research probably could be the production of marine PL liposomes without using organic solvent by microfluidization for food applications.

LIST OF REFERENCES

- Ahmad, I., Alaiz, M., Hidalgo, F. J., & Zamora, R. (1998). Effect of oxidized lipid/amino acid reaction products on the antioxidative activity of common antioxidants. *Journal of Agricultural and Food Chemistry*, 46, 3768-3771.
- Aidos, I., Jacobsen, C., Jensen, B., Luten, J. B., Padt, A. V. D., & Boom, R. M. (2002). Volatile oxidation products formed in crude herring oil under accelerated oxidative conditions. *European Food Research and Technology*, 104, 808-818.
- Alaiz, M., Hidalgo, F. J., & Zamora, R. (1995a). Antioxidative activity of (E)-2-octenal/amino acids reaction products. *Journal of Agricultural and Food Chemistry*, 43, 795-800.
- Alaiz, M., Hidalgo, F. J., & Zamora, R. (1995b). Natural antioxidants produced in oxidized lipid/amino acid browning reactions. *Journal of the American Oil Chemists' Society*, 72, 1571-1575.
- Alaiz, M., Hidalgo, F. J., & Zamora, R. (1995c). Addition of oxidized lipid/amino acid reaction products delays the peroxidation initiated in a soybean oil. *Journal of Agricultural and Food Chemistry*, 43, 2698-2701.
- Alaiz, M., Hidalgo, F. J., & Zamora, R. (1996). Contribution of the formation of oxidized lipid/amino acid reaction products to the protective role of amino acids in oils and fats. *Journal of Agricultural and Food Chemistry*, 44, 1890-1895.
- American Heart Association (2002). AHA scientific statement: fish consumption, fish oil, omega-3 fatty acids and cardiovascular disease. *Circulation*, 106, 2747-2757.
- Anese, M., & Nicoli, M. C. (2003). Antioxidant properties of ready-to drink coffee brews. *Journal of Agricultural and Food Chemistry*, 51, 942-946.
- Applegate, K. R., & Glomset, J. A. (1986) Computer-Based Modeling of the Conformation and Packing Properties of Docosahexaenoic Acid. *Journal of Lipid Research*, 27, 658-680.
- Arts, T. J. C., Laven, J., Vader, F. V., & Kwaaitaal, T. (1994). Zeta potentials of tristeroylglycerol crystals in olive oil, *Colloid Surfaces A: Physicochemical and Engineering Aspects*, 85, 149-158.
- Asai, Y. (2003) Formation of dispersed particles composed of soybean oil and phosphatidyl choline. *European Journal of Lipid Science and Technology*, 105, 397-402.

- Asai, Y., & Watanabe, S. (1999) Interaction of sesame oil with soybean phosphatidylcholine and their formation of small dispersed particles. *Journal of Microencapsulation*, 16, 705-713.
- Bandarra, N. M., Campos, R. M., Batista, I., Nunes, M. L., & Empis, J. M. (1999) Antioxidant synergy of alpha-tocopherol and phospholipids. *Journal of the American Oil Chemists' Society*, 76, 905-913.
- Baynes, J. W., Monnier, V. M., & Ames, J. M. (2005). *The Maillard Reaction. Chemistry at the interface of nutrition, aging and disease.* The New York Academy of Sciences, New York, NY.
- Belhaj, N., Arab-Tehrany, E., & Linder, M. (2010). Oxidative kinetics of salmon oil in bulk and in nanoemulsion stabilized by marine lecithin. *Process biochemistry*, 45, 187-195.
- Berger, K. G., & Hamilton, R. J. (1995). Lipids and oxygen: Is rancidity avoidable in practice? In: Hamilton RJ (ed) *Development in oils and fats.* Glasgow, UK: Blackie Academic & professional.
- Bueschelberger, H. G. (2004) Lecithin. In: Whitehurst RJ (ed) *Emulsifiers in Food Technology.* Oxford, UK: Blackwell Publishing Ltd.
- Buszello, K., Harnisch, S., Muller, R. H., & Muller, B. W. (2000). The influence of alkali fatty acids on the properties and the stability of parenteral O/W emulsions modified with Solutol HS 15 (R). *European Journal of Pharmaceutics Biopharmaceutics*, 49, 143-149.
- Body, D. R., & Vlieg, P. (1989) Distribution of the Lipid Classes and Eicosapentaenoic (20-5) and Docosahexaenoic (22-6) Acids in Different Sites in Blue Mackerel (*Scomber Australasicus*) Fillets. *Journal of Food Science*, 54, 569-572.
- Boon, C., McClements, D., Weiss, J., & Decker, E. (2009). Role of iron and hydroperoxides in the degradation of lycopene in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, 57, 2993-2998.
- Boyd, L. C., Nwosu, V. C., Young, C. L., & MacMillian, L. (1998) Monitoring lipid oxidation and antioxidant effects of phospholipids by headspace gas chromatographic analyses of rancimat trapped volatiles. *Journal of Food Lipids*, 5, 269-282.
- British Nutrition Foundation's Task Force (1992). *Unsaturated fatty acids: Nutrition and Physiological Significance*, Chapman and Hall, London, UK.
- Cansell, M., Moussaoui, N., & Lefrancois, C. (2001) Stability of marine lipid based liposomes under acid conditions. Influence of xanthan gum. *Journal of Liposome Research*, 11, 229-242.
- Cercaci, L., Rodriguez-Estrada, M. T., Lercker, G., & Decker, E. A. (2007). Phytosterol oxidation in oil-in-water emulsions and bulk oil. *Food Chemistry*, 102, 161-167.

Chaiyasit, W., Elias, R., McClements, D. J., & Decker, E. A. (2007). Role of physical structures in bulk oils on lipid oxidation. *Critical Reviews in Food Science and Nutrition*, 47, 299-317.

Chee, C. P., Roberts, R. F., & Coupland, J. N. (2006). Effect of temperature, time, medium form and casein on lipid oxidation of polyunsaturated fatty acids in algae oil. *Milchwissenschaft-Milk Science International*, 61, 142-145.

Chung, H. Y. (1999). Volatile compound in cranmeats of *Charybdis feriatius*. *Journal of Agricultural and Food Chemistry*, 47, 6, 2280-2287.

Chung, H. Y., Yung I. K. S., & Kim J. S. (2001). Comparison of volatile components in dried scallops (*Chlamys farreri* and *Patinopecten yessoensis*) prepared by boiling and steaming methods. *Journal of Agricultural and Food Chemistry*, 49, 192-202.

Cho, S. Y., Joo, D. S., Choi, H. G., Nara, E., & Miyashita, K. (2001) Oxidative stability of lipids from squid tissues. *Fisheries Science*, 67, 738-743.

Coupland, J. N., & McClements, D. J. (1996). Lipid oxidation in food emulsions. *Trends in Food Science and Technology*, 7, 83-91.

Dalton, A., Witthuhn, R. C., Smuts, C. M., Walmarans, P., & Nel, D. G. (2006). Development, microbiological content and sensory analysis of a spread rich in n-3 fatty acids. *Food Research International*, 39, 559-567.

Dobarganes, M. C., & G. Marquez-Ruiz. (2007). Formation and analysis of oxidized monomeric, dimeric and higher oligomeric triglycerides. In: Erickson MD (ed) *Deep frying: Chemistry, Nutrition and Practical Applications*. Champaign, IL: AOCS Press.

Domingues, M. R. M., Reis, A., & Domingues, P. (2008) Mass spectrometry analysis of oxidized phospholipids. *Chemistry and Physics of Lipids*, 156, 1-12.

Dunford, H. B. (1987). Free radicals in iron containing systems. *Free Radical Biology and Medicine*, 3, 405-421.

EFSA (2010) EFSA sets European dietary references values for nutrient intakes. (www.efsa.europa.eu/en/press/news/nda100326.htm).

Erickson, M. C. (2008). Chemistry and function of phospholipids. In: Akoh CC & Min DB (ed) *Food lipids: Chemistry, Nutrition and Biotechnology*. Boca Raton, FL: CRC Press.

Falch, E., Rustad, T., Jonsdottir, R., Shaw, N. B., Dumay, J., Berge, J. P., Arason, S., Kerry, J. P., Sandbakk, M., & Aursand, M. (2006) Geographical and seasonal differences in lipid composition and relative weight of by-products from gadiform species. *Journal of Food Composition and Analysis*, 19, 727-736.

FDA (2008a). U. S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Food Additives Safety: Agency Response Letter: GRAS Notice No. GRN 000226, January 3, 2008.

FDA (2008b). U. S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Food Additives Safety: Agency Response Letter: GRAS Notice No. GRN 000242, October 14, 2008.

FDA (2011). U. S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Food Additives Safety: Agency Response Letter: GRAS Notice No. GRN 000371, July 22, 2011.

Finean, J. B. (1990) Interaction Between Cholesterol and Phospholipid in Hydrated Bilayers. *Chemistry and Physics of Lipids*, 54, 147-156.

Fiorentini, D., Landi, L., Barzanti, V., & Cabrini, L. (1989) Buffers can modulate the effect of sonication on egg lecithin liposomes. *Free Radical Research Communications*, 6, 243-250

Flores, M., Spanier, A. M., & Toldra, F. (1998). Flavour analysis of dry-cured ham. In: Shahidi F (ed) *Flavour of meat and meat products and seafoods*. London, UK: Blackie Academic and Professional.

Frankel, E. N. (2005). *Lipid Oxidation*. Bridgwater, England: The Oily Press.

Frankel, E. N., Huang, S., Aeschbach, R., & Prior, E. (1996a). Antioxidant activity of a rosemary extract and its constituents, carnosic acid, carnosol and rosmarinic acid in bulk oil and oil-in water emulsion. *Journal of Agricultural and Food Chemistry*, 44, 131-135.

Frankel, E. N., Huang, S., Aeschbach, R., & Prior, E. (1996b). Evaluation of antioxidant activity of a rosemary extract, carnosol and carnosic acid in bulk vegetable oils and fish oils and their emulsions. *Journal of the Science of Food and Agriculture*, 72, 201-208.

Garcia, E., Gutierrez, S., Nolasco, H., Carreon, L., & Arjona, O. (2006). Lipid composition of shark liver oil: effects of emulsifying and microencapsulation processes. *European Food Research and Technology*, 222, 697-701.

Gbogouri, G. A., Linder, M., Fanni, J., & Parmentier, M. (2006). Analysis of lipids extracted from salmon (*Salmo salar*) heads by commercial proteolytic enzymes, *European Food Research and Technology*, 108, 766-775.

Gritt M., Zuidam N. J., Underberg, W. J. M., & Crommelin, D. J. A. (1993) Hydrolysis of partially saturated egg phosphatidylcholine in aqueous liposome dispersions and the effect of cholesterol incorporation in hydrolysis kinetics. *Journal of Pharmacy and Pharmacology*, 45, 490-495.

Halliwell, B., & Gutteridge, J. (1990). Role of free radicals and catalytic metal ions in human disease: an overview. *Methods in Enzymology*, 186, 1-85.

Hartvigsen, K., Lund, P., Hansen, L. F., & Hølmer, 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, 4858-4867.

Herman, C. J., & Groves, M. J. (1992). Hydrolysis Kinetics of Phospholipids in Thermally Stressed Intravenous Lipid Emulsion Formulations. *Journal of Pharmacy and Pharmacology*, 44, 539-542.

Hidalgo, F., Alaiz, M., & Zamora, R. (1999). Effect of pH and temperature on comparative non-enzymatic browning of proteins produced by oxidized lipids and carbohydrate. *Journal of Agricultural and Food Chemistry*, 47, 742-747.

Hidalgo, F. J., Mercedes leon, M., & Zamora, R. (2006). Antioxidative activity of amino phospholipids and phospholipid/amino acid mixtures in edible oils as determined by the Rancimat method. *Journal of Agricultural and Food Chemistry*, 54, 5461-5467.

Hidalgo, F. J., Mercedes leon, M., Nogales, F., & Zamora, R. (2007) Effect of Tocopherols in the Antioxidative Activity of Oxidized Lipid-Amine Reaction Products. *Journal of Agricultural and Food Chemistry*, 55, 4436-4442.

Hidalgo, F. J., Nogales, F., & Zamora, R. (2003). Effect of the pyrrole polymerization mechanism on the antioxidative activity of nonenzymatic browning reactions. *Journal of Agricultural and Food Chemistry*, 51, 5703-5708.

Hidalgo, F. J., Nogales, F. & Zamora, R. (2005a). Nonenzymatic Browning, Fluorescence development, and formation of pyrrole derivatives in phosphatidylethanolamine/Ribose/Lysine model systems. *Journal of Food Science*, 70, 387-391.

Hidalgo, F. J., Nogales, F., & Zamora, R. (2005b). Changes produced in the antioxidative activity of phospholipids as a consequence of their oxidation. *Journal of Agricultural and Food Chemistry*, 53, 659-662.

Hidalgo, F. J. & Zamora, R. (1993). Fluorescent pyrrole products from carbonyl-amine reactions. *Journal of Biological Chemistry*, 268, 16190-16197.

Hidalgo, F. J. & Zamora, R. (2004). Strecker -type degradation produced by the lipid oxidation products 4, 5-epoxy-2-alkenals. *Journal of Agriculture and Food chemistry*, 52, 7126-7131.

Hidalgo, F. J. & Zamora, R. (2005). Interplay between the Maillard reaction and lipid peroxidation in biochemical systems, *Annual New York Academic Sciences*, 1043, 319-326.

Hussein, N., Ah Sing, E., Wilkinson, P., Leach, C., Griffin, B. A., & Millward, D. J. (2005). Relative rates of long chain conversion of ¹³C linoleic and α -linolenic acid in response to marked changes in their dietary intake in male adults. *Journal of Lipid Research*, 46, 269-280.

Ierna, M., Kerr, A., Scales, H., Berge, K., & Griinari, M. (2010) Supplementation of diet with krill oil protects against experimental rheumatoid arthritis. *BMC Musculoskeletal Disorders*, 11.

Israelachvili, J.N. (1992). *Intermolecular and Surface Forces*. London, UK: Academic Press.

Israelachvili, J.N. (1994). The science and applications of emulsions—an overview. *Colloids and Surfaces*, 91, 1.

ISSFAL Board Statement (2004) Recommendations for Intake of Polyunsaturated Fatty Acids in Healthy Adults, International Society for the Study of Fatty Acids and Lipids (www.issfal.org.uk/lipid-matters/issfal-policy-statements/issfal-policy-statement-3.html).

Jacobsen, C., Timm-Heinrich, M., & Meyer, A. (2001). Oxidation in fish oil enriched mayonnaise: ascorbic acid and low pH increase oxidative deterioration. *Journal of Agricultural and Food Chemistry*, 49, 3947-3956.

Jacobsen, C., Xu, X., Nielsen, N. S., & Timm-Heinrich, M. (2003). Oxidative stability of mayonnaise containing structured lipids produced from sunflower oil and caprylic acid. *European Journal of Lipid Science and Technology*, 105, 449-458.

Koga, T., & Terao, J. (1995). Phospholipids increase radical scavenging activity of vitamin E in a bulk oil model system. *Journal of Agricultural and Food Chemistry*, 43, 1450-1454.

Karahadian, C., & Lindsay, R. C. (1989). Evaluation of compounds contributing characterizing fishy flavours in fish oil. *Journal of the American Oil Chemists' Society*, 66, 953-960.

Kassis, N. M., Beamer, S. K., Matak, K. E., Tou, J. C., & Jaczynski, J. (2010). Nutritional composition of novel nutraceutical egg products developed with omega-3-rich oils. *LWT-Food Science and Technology*, 43, 1204-1212.

Kassis, N. M., Gigliotti, J. C., Beamer, S. K., Tou, J. C., & Jaczynski, J. (2011). Characterization of lipids and antioxidant capacity of novel nutraceutical egg products developed with omega-3-rich oils. *Journal of the Science of Food and Agriculture*, 92, 66-73.

Kashima, M., Cha, G.S., Isoda, Y., Hirano, J., & Miyazawa, T. (1991) The Antioxidant Effects of Phospholipids on Perilla Oil. *Journal of the American Oil Chemists' Society*, 68, 119-122.

Khayat, A., & Schwall, D. (1983). Lipid oxidation in seafood. *Food Technology*, 37, 130-1400.

Kim, I. H., Kim, C. J., & Kim, D. H. (1999). Physicochemical properties of methyl linoleate oxidized at various temperatures. *Korean Journal of Food Science and Technology*, 31, 600-605.

King, M. F., Boyd, L. C., & Sheldon, B. W. (1992a) Effects of Phospholipids on Lipid Oxidation of A Salmon Oil Model System. *Journal of the American Oil Chemists' Society* 69, 237-242.

King, M. F., Boyd, L. C., & Sheldon, B. W. (1992b) Antioxidant Properties of Individual Phospholipids in A Salmon Oil Model System. *Journal of the American Oil Chemists' Society*, 69, 545-551.

- Kolanowski, W., & Laufenberg, G. (2006). Enrichment of food products with polyunsaturated fatty acids by fish oil addition. *European Food Research and Technology*, 22, 472-477.
- Kolanowski, W., Laufenberg, G., & Kunz, B. (2004). Fish oil stabilization by microencapsulation with modified cellulose. *International Journal of Food Science and Nutrition*, 55, 333-343.
- Kulas, E., Olsen, E., & Ackman, R. G. (2003). Oxidation of fish lipids and its inhibition with tocopherols. In: Kamal EA (Ed), *Lipid oxidation pathway*. Champaign, IL: AOCS Press
- Laguerre, M., Lopez Giraldo, L., Lecomte, J., Figueroa-Espinoza, M., Barea, B., Weiss, J., Decker, E. A., & Villeneuve, P. (2009). Chain length affects antioxidant properties of chlorogenate esters in emulsion: the cut off theory behind the polar paradox. *Journal of Agricultural and Food Chemistry*, 57, 11335-11342.
- Le Grandois, J., Marchioni, E., Zhao, M. J., Giuffrida, F., Ennahar, S., & Bindler, F. (2009) Investigation of Natural Phosphatidylcholine Sources: Separation and Identification by Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry (LC-ESI-MS2) of Molecular Species, *Journal of Agricultural and Food Chemistry*, 57, 6014-6020.
- Let, M. B., Jacobsen, C., Sorensen, A. D., & Meyer, A. S. (2007). Homogenisation condition affects the oxidative stability of fish oil enriched milk emulsions. *Journal of Agricultural and Food Chemistry*, 51, 1773-1780.
- Linder, M., & Ackman, R. G. (2002). Volatile compounds recovered by Solid Phase Microextraction from fresh adductor muscle and total lipids of sea scallop (*Placopecten magellanicus*) from Georges Bank (Nova Scotia). *Journal of Food Science*, 67, 2032-2037.
- Lu, F. S. H., & Norziah, M. H. (2010). Stability of Docosahexaenoic acid (DHA) and eicosapentanoic acid (EPA) in breads after baking and upon storage. *International Journal of Food Science and Technology*, 45, 821-827.
- Lu, F. S. H., & Norziah, M. H. (2011). Contribution of microencapsulated n-3 PUFA powder toward sensory and oxidation stability of bread. *Journal of Food Processing & Preservation*, 35, 596-604.
- Mancuso, J. R., McClement, D. J., & Decker, E. A. (1999). Ability of iron to promote surfactant peroxide decomposition and oxidize alpha-tocopherol. *Journal of Agricultural and Food Chemistry*, 47, 4146-4149.
- McClements, D. J. (2005). *Food Emulsions: Principles, Practices, and Techniques*. Boca Raton, FL: CRC Press.
- 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, 1271-1282.

Medina, I., Aubourg, S. P., & Martin, R. P. (1995) Composition of Phospholipids of White Muscle of 6 Tuna Species. *Lipids* 30, 1127-1135.

Mei, L. Y., Decker, E. A., & McClements, D. J. (1998a) Evidence of iron association with emulsion droplets and its impact on lipid oxidation. *Journal of Agricultural and Food Chemistry*, 46, 5072-5077.

Mei, L. Y., McClements, D. J., Wu, J., & Decker, E. A. (1998b). Iron catalyzed lipid oxidation in emulsion as affected by surfactant, pH, NaCl. *Food chemistry*, 61, 307-312.

Minotti, G., & Aust, S. (1989). The role of iron in oxygen radical mediated lipid peroxidation. *Chemico- Biological Interactions*, 71, 1-19.

Miyashita, K., Nara, E., & Ota, T. (1993). Oxidation stability of polyunsaturated fatty acids in aqueous solution. *Bioscience, Biotechnology, and Biochemistry*, 57, 1638-1640.

Miyashita, K., Nara, E., & Ota, T. (1994). Comparative-Study on the Oxidative Stability of Phosphatidylcholines from Salmon Egg and Soybean in An Aqueous-Solution. *Bioscience, Biotechnology, and Biochemistry*, 58, 1772-1775.

Mohammad, A., Olcott, H. S., & Fraenkel-Conrat, H. (1946). Reaction of protein with acetaldehyde. *Archives of Biochemistry and Biophysics*, 24, 270-280.

Monroig O., Navarro, J. C., Amat, I., Gonzalez, P., Amat, F., & Hontoria, F. (2003). Enrichment of *Artemia* nauplii in PUFA, phospholipids, and water-soluble nutrients using liposomes. *Aquaculture International* 1, 151–161

Moriya, H., Kuniminato, T., Hosokawa, M., Fukunaga, K., Nishiyama, T., & Miyashita, K. (2007). Oxidative stability of salmon and herring roe lipids and their dietary effect on plasma cholesterol levels of rats. *Fisheries Science*, 73, 668–674.

Mozafari, M. R., Khosravi-Darani, K., Borazan, G. G., Cui, J., Pardakhty, A., & Yurdugul, S. (2008). Encapsulation of Food Ingredients Using Nanoliposome Technology. *International Journal of Food Properties*, 11, 833-844.

Mozuraityte, R., Rustad, T., & Storro, I. (2006a) Pro-oxidant activity of Fe²⁺ in oxidation of cod phospholipids in liposomes. *European Journal of Lipid Sciences and Technology*, 108, 218-226.

Mozuraityte, R., Rustad, T., & Storro, I. (2006b) Oxidation of cod phospholipids in liposomes: Effects of salts, pH and zeta potential. *European Journal of Lipid Sciences and Technology*, 108, 944-950.

Mozuraityte, R., Rustad, T., & Storro, I. (2008). The role of iron in peroxidation of polyunsaturated fatty acids in liposomes. *Journal of Agricultural and Food Chemistry*, 56, 537-543.

Nacka, F., Cansell, M., Meleard, P., & Combe, N. (2001a). Incorporation of alpha tocopherol in marine lipid-based liposomes: in vitro and in vivo studies. *Lipids* 36, 1313-1320.

- Nacka, F., Cansell, M., Gouygou, J. P., Gerbeaud, C., Meleard, P., & Entressangles, B. (2001b). Physical and chemical stability of marine lipid-based liposomes under acid conditions. *Colloid Surfaces B*, 20, 257-266.
- Nara, E., Miyashita, K., & Ota, T. (1997). Oxidative stability of liposomes prepared from soybean PC, chicken egg PC, and salmon egg PC. *Bioscience, Biotechnology, and Biochemistry*, 61, 1736-1738.
- Nara, E., Miyashita, K., Ota, T., & Nadachi, Y. (1998). The oxidative stabilities of polyunsaturated fatty acids in salmon egg phosphatidylcholine liposomes. *Fisheries Sciences*, 64, 282-286.
- Neptune Technologies & Bioresources. Natural phospholipids of marine origin containing flavonoids and polyunsaturated phospholipids and their uses. [EP 1417211]. 2001.
- Nielsen, N. S. Debnath, D., & Jacobsen, C. (2007). Oxidative stability of fish oil enriched drinking yoghurt. *International Dairy Journal*, 17, 1478-1485.
- Pawlosky, R. J., Hibbeln, J. R., Novotny, J. A., & Salem, N. (2001). Physiological compartmental analysis of α -linoleic acid metabolism in adult humans. *Journal of Lipid Research*, 42, 1257-1265.
- Peng, J. L., Larondelle, Y., Pham, D., Ackman, R. G., & Rollin, X. (2003). Polyunsaturated fatty acid profiles of whole body phospholipids and triacylglycerols in anadromous and landlocked Atlantic salmon (*Salmo salar* L.) fry. *Comparative Biochemistry and Physiology Part B*, 134, 335-348.
- Pietrowski, B. N., Tahergorabi, R., Matak, K. E., Tou, J. C., & Jaczynski, J. (2011). Chemical properties of surimi seafood nutrified with ω -3 rich oils. *Food Chemistry*, 129, 912-919.
- Pokorny, J., & Sakurai, H. (2002). Role of oxidized lipids in nonenzymatic browning reactions. *International Congress Series*, 1245, 373-374.
- Pripis-Nicolau, L., Revel, G. D., Bertrand, A., & Maujean, A. (2000). Formation of flavor components by the reaction of amino acid and carbonyl compounds in mild conditions. *Journal of Agricultural and Food Chemistry*, 48, 3762-3766.
- Reineccius, G. (2006). Changes in food flavor due to processing. In: *Flavor chemistry and Technology*. Boca Raton, FL: Taylor & Francis group.
- Reische, D. W., Lillard, D. A., & Eitenmiller, R. R. (1998). Antioxidants. In: Akoh CC & Min DB (ed) *Food lipids: Chemistry, Nutrition and Biotechnology*. Boca Raton, FL: CRC Press.
- Rudolph, M. J. (2001). A scoopful of nutrition: Enriching ice-cream with fish oil. *Innovations Food Technology*, 13, 69-70.

Saito, H., Kotani, Y., Keriko, J.M., Xue, C.H., Taki, K., Ishihara, K., Ueda, T., & Miyata, S. (2002). High levels of n-3 polyunsaturated fatty acids in *Euphausia pacifica* and its role as a source of docosahexaenoic and eicosapentaenoic acids for higher trophic levels, *Marine Chemistry*, 78, 9-28.

Sasaki, K., Alamed, J., Weiss, J., Villeneuve, P., Lopez Giraldo, L., Lecomte, J., Figueroa-Espinoza, M. C., & Decker, E. A. (2010). Relationship between the physical properties of chlorogenic acid esters and their ability to inhibit lipid oxidation in oil-in-water emulsions. *Food Chemistry*, 118, 830-835.

Schneider, M. (2008). Major sources, composition and processing. In: Gunstone FD (ed) *Phospholipid Technology and Applications*. Bridgwater, England: The Oily Press.

Schneider M., & Lovaas, E. (2009). Process for the production of phospholipids. US2009/0028989.

Striby, L., Lafont, R., & Goutx, M. (1999). Improvement in the Iatroscan thin-layer chromatographic-flame ionisation detection analysis of marine lipids. Separation and quantitation of monoacylglycerols and diacylglycerols in standards and natural samples, *Journal of Chromatography A*, 849, 371-380.

Sedoski, H. D., Beamer, S. K., Jaczynski, J., Partington, S., & Matak, K. E. (2012). Sensory evaluation and quality indicators of nutritionally enhanced egg products with ω -3 rich oils. *LWT-Food Science and Technology*, 47, 459-464.

Sørensen, A. D., Haahr, A., Becker, E., Skibsted, L., Bergenstahl, B., Nilsson, L., & Jacobsen, C. (2008). Interactions between iron, phenolic compounds, emulsifiers, and pH in omega-3 enriched oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, 56, 1740-1750.

Sørensen, A. D. M., Nielsen, N. S., Hyldig, G., & Jacobsen, C. (2010a). The influence of emulsifier type on lipid oxidation in fish oil enriched light mayonnaise. *European Food Research and Technology*, 112, 476-487.

Sørensen, A. D. M., Nielsen, N. S., & Jacobsen, C. (2010b). Oxidative stability of fish oil enriched mayonnaise based salads. *European Food Research and Technology*, 112, 476-487

Tadolini, B., & Hakim, G. (1996). The mechanism of iron (III) stimulation of lipid peroxidation. *Free Radical Research*, 25, 221-227.

Thanonkaew A., Benjakul S., Visessanguan W., & Decker, E. A. (2005). Lipid oxidation in microsomal fraction of squid muscle (*Loligo peali*). *Journal of Food Science*, 70, 478-482.

Thanonkaew A., Benjakul S., & Visessanguan W. (2006a). Chemical composition and thermal property of cuttlefish (*Sepia pharaonis*) muscle. *Journal of Food Composition and Analysis*, 19, 127-133.

- Thanonkaew A., Benjakul S., Visessanguan W., & Decker, E. A. (2006b). Development of yellow pigmentation in squid (*Loligo peali*) as a result of lipid oxidation. *Journal of Agricultural and Food Chemistry*, 54, 956-962.
- Thanonkaew A., Benjakul S., Visessanguan W., & Decker, E. A. (2007). Yellow discoloration of the liposome system of cuttlefish (*Sepia pharaonis*) as influenced by lipid oxidation. *Food Chemistry*, 102, 219-2240.
- Thompson, A. K., Hindmarsh, J. P., Haisman, D., Rades, T., & Singh, H. (2006). Comparison of the structure and properties of liposomes prepared from milk fat globule membrane and soy phospholipids. *Journal of Agricultural and Food Chemistry*, 54, 3704-3711.
- Tompkins, C., & Perkins, E. G. (2000). Frying performance of low linolenic acid soybean oil. *Journal of the American Oil Chemists' Society*, 77, 223-229.
- Trautwein, E. A. (2001). n-3 Fatty acids – physiological and technical aspects for their use in Food. *European Journal of Lipid Science and Technology*, 103, 45-55.
- Uematsu, T., Parkanyiova, L., Endo, T., Matsuyama, C., Yano, T., Mitsuyoshi, M., Sakurai, H., & Pokorny, J. (2002). Effect of the unsaturation degree on browning reactions of peanut oil and other edible oils with proteins under storage and frying conditions. *International Congress Series*, 1245, 445-446.
- Venkateshwarlu, G., Let, M. B., Meyer, A. S., & Jacobsen, C. (2004). Chemical and olfactometric characterization of volatile flavour compounds in a fish oil enriched milk emulsion. *Journal of Agricultural and Food Chemistry*, 52, 311-317.
- Ventanas, S., Estevez, M., & Delgado, C. L. (2007). Phospholipid oxidation, non-enzymatic browning development and volatile compounds generation in model systems containing liposomes from porcine *Longissimus dorsi* and selected amino acids. *European Food Research and Technology*, 225, 665-675.
- Verardo, V., Ferioli, F., Riciputi, Y., Lafelice, G., Marconi, E., & Carboni, M. F. (2009). Evaluation of lipid oxidation in spaghetti pasta enriched with long chain n-3 fatty acids under different storage conditions. *Food Chemistry*, 114, 472-477.
- Waraho, T., McClement, D. J., & Decker, E. A. (2011). Mechanisms of lipid oxidation in food dispersions. *Trends in Food Science and Technology*, 22, 3-13.
- Weng, X. C., & Gordon, M. H. (1993) Antioxidant Synergy Between Phosphatidyl Ethanolamine and Alpha-Tocopherylquinone, *Food Chemistry*, 48, 165-168.
- Whitfield, F. B. (1992). Volatiles from interaction of Maillard reactions and lipids. *Critical Review in Food Science and Nutrition*, 31, 1-58.
- Whelan, J., & Rust, C. (2006). Innovative dietary sources of n-3 fatty acids. *Annual Review of Nutrition*, 26, 75-103.

Wijendran, V., Huang, M. C., Diau, G. Y., Boehm, G., Nathanielsz, P. W., & Brenna, J. T. (2002) Efficacy of dietary arachidonic acid provided as triglyceride or phospholipid as substrates for brain arachidonic acid accretion in baboon neonates. *Pediatric Research*, 51, 265-272.

Wong, J. M., & Berndhard, R. A. (1998). Effect of nitrogen source on pyrazine formation. *Journal of Agricultural and Food Chemistry*, 36, 123-129.

Yu, H. Z., & Chen, S. S. (2010). Identification of characteristic aroma-active compounds in steamed mangrove crab (*Scylla serrata*). *Food Research International*, 43, 2081-2086.

Yuji, H., Weiss, j., Villeneuve, P., Lopez Gilraldo, L., Figueroa-Espinoza, M., & Decker, E. A. (2007). Ability of striface active antioxidant in oil-in-water emulsion. *Journal of Agricultural and Food Chemistry*, 55, 11052-11056.

Zamora, R., Alaiz, M., & Hidalgo, F. J. (2000). Contribution of pyrrole formation and polymerization to the nonenzymatic browning produced by amino-carbonyl reactions. *Journal of Agricultural and Food Chemistry*, 48, 3152-3158.

Zamora, R., Gallardo, E., & Hidalgo, F. J. (2006). Chemical conversion of α -amino acids into α -keto acids by 4, 5-epoxy-2-decenal. *Journal of Agricultural and Food Chemistry*, 54, 6101-6105.

Zamora, R., Gallardo, E., & Hidalgo, F. J. (2007). Strecker degradation of phenylalanine initiated by 2, 4-decadienal or methyl 13-oxooctadeca-9, 11-dienoate in model systems. *Journal of Agricultural and Food Chemistry*, 55, 1308-1314.

Zamora, R., & Hidalgo, F. J. (1994). Modification of lysine amino groups by the lipid peroxidation product 4, 5(E)-epoxy-2(E)-heptenal. *Lipids*, 29, 243-249.

Zamora, R., & Hidalgo, F. J. (1995). Linoleic acid oxidation in the presence of amino compounds produces pyrroles by carbonyl amine reactions. *Biochimica et Biophysica Acta*, 1258, 319-327.

Zamora, R., & Hidalgo, F. J. (2005). Coordinate contribution of lipid oxidation and Maillard reaction to the nonenzymatic food browning. *Critical reviews in Food Science and Nutrition*, 45, 49-59.

Zamora, R., & Hidalgo, F. J. (2011). The Maillard reaction and lipid oxidation. *Lipid Technology*, 23, 59-62.

Zamora, R., Nogales, F., & Hidalgo, F. J. (2005). Phospholipid oxidation and nonenzymatic browning development in phosphatidylethanolamine/ ribose/lysine model systems. *European Food Research and Technology*, 220, 459-465.

Zamora, R., Olmo, C., Navarro, J. L., & Hidalgo, F. J. (2004). Contribution of phospholipid pyrrolization to the color reversion produced during deodorization of poorly degummed vegetable oils. *Journal of Agricultural and Food Chemistry*, 52, 4166-4171.

APPENDIX

PAPER I

Lu, F. S. H., Nielsen, N. S., Heinrich, M. T., Jacobsen, C.

**Oxidative stability of marine phospholipids in the liposomal form and their applications:
A review**

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Oxidative Stability of Marine Phospholipids in the Liposomal Form and Their Applications

F. S. Henna Lu · N. S. Nielsen · M. Timm-Heinrich · C. Jacobsen

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Abstract Marine phospholipids (MPL) have attracted a great deal of attention recently as they are considered to have a better bioavailability, a better resistance towards oxidation and a higher content of eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) than oily triglycerides (fish oil) from the same source. Due to their tight intermolecular packing conformation at the *sn*-2 position and their synergism with α -tocopherol present in MPL extracts, they can form stable liposomes which are attractive ingredients for food or feed applications. However, MPL are still susceptible to oxidation as they contain large amounts polyunsaturated fatty acids and application of MPL in food and aquaculture industries is therefore a great challenge for researchers. Hence, knowledge on the oxidative stability of MPL and the behavior of MPL in food and feed systems is an important issue. For this reason, this review was undertaken to provide the industry and academia with an overview of (1) the stability of MPL in different forms and their potential as liposomal material, and (2) the current applications and future prospects of MPL in both food and aquaculture industries with special emphasis on MPL in the liposomal form.

Keywords Marine phospholipids · Antioxidants · n-3 PUFA · Eicosapentaenoic acid · Docosahexaenoic acid · Oxidative stability · *sn*-2 Position · Liposome · Food industry · Aquaculture industry

Abbreviations

AA	Arachidonic acid
BHT	Butylated hydroxytoluene
CHO	Cholesterol
CL	Cardiolipin
DAG	Diacylglycerols
DHA	Docosahexaenoic acid
DP	Diacetyl phosphate
EE	Encapsulation efficiency
EFA	Essential fatty acid
EPA	Eicosapentaenoic acid
LA	Linoleic Acid
LPC	Lysophosphatidylcholine
LUV	Large unilamellar vesicles
MLV	Multilamellar vesicles
MPL	Marine phospholipids
n-3 PUFA	Omega-3 polyunsaturated fatty acid(s)
PA	Palmitic acid
PC	Phosphatidylcholine(s)
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PL	Phospholipid(s)
PS	Phosphatidylserine
SA	Stearylamine
SPM	Sphingomyelin
TAG	Triacylglycerols
TL	Total lipids
NL	Neutral lipids

F. S. Henna Lu · N. S. Nielsen · C. Jacobsen (✉)
Division of Seafood Research, Lipids and Oxidation Group,
National Food Institute, Technical University of Denmark,
Søltøfts Plads, Building 221, 2800 Kgs. Lyngby, Denmark
e-mail: cja@food.dtu.dk

F. S. Henna Lu
e-mail: fshl@food.dtu.dk

M. Timm-Heinrich
BASF A/S, Production unit Ballerup,
Malmparken 5, 2750 Ballerup, Denmark

Introduction

The present imbalance in the intake of n-3 and n-6 polyunsaturated fatty acids (PUFA) has a serious negative impact on health in the general population [1–3] and there is a strong desire to improve the situation by introducing new products on the market with a higher level of n-3 PUFA and a lower level of n-6 PUFA. Currently, the global food and dietary supplement market for n-3 fatty acids (EPA and DHA) is estimated to be 15,000–20,000 tons, derived from a total world production of fish oil of approximately 300,000 tons per year. Marine phospholipids (MPL) from, e.g., krill represents an alternative source of n-3 PUFA, but the market for MPL is still in its infancy even though an increasing activity in this field has been observed recently [4]. A number of companies are preparing market introduction of either natural MPL, derivatives of natural MPL, or synthetic MPL. The leading MPL product on the market at the moment is a krill extract with approximately 35% PL [5]. There are also MPL products that are made from fish processing by-products and salmon roe. It is expected that the MPL market will follow the general trends of n-3 fish oils. MPL are new on the market and their range of applications has yet to be determined. However, MPL are believed to have potential applications in human and animal nutrition, in pharmacology, and in drug delivery. The most well-documented applications of MPL are related to liposomes. Liposomes made from MPL have been developed as a test system for antioxidants and as model systems for oxidation of biological membranes [6–9].

Many studies have been performed on n-3 triacylglycerols (TAG) enriched functional foods [10] while limited studies have been carried out on MPL enriched functional foods either in their pure form or in liposomal form. Furthermore, the current applications of phospholipid liposomes are limited to lecithin from soy bean or phosphatidylcholine (PC) from egg yolk and no attempts to use MPL based liposomes for food purposes have been reported in the literature [11–13]. However, some studies [14–19] have investigated the use of MPL such as herring roe or krill PL for larvae feed in the aquaculture industry. The limited application of MPL and liposomes in both food and aquaculture industries can be attributed to several reasons (1) lack of knowledge especially related to the behavior of MPL in food and feed systems, (2) limitations in large scale production of liposomes without using organic solvents and (3) the requirement of expensive equipment for liposome production. Nevertheless, there is ongoing research in this area [20–28]. With the growing understanding of the following areas regarding (1) the physicochemical properties of MPL, (2) the oxidative stability of MPL or MPL based liposomes under gastrointestinal condition and (3)

emerging technologies for liposome production without using organic solvents such as microfluidization and pro-liposomes method [29], it may soon become feasible to use MPL in both the food and aquaculture industries. This review gives an overview of our current knowledge on the above mentioned aspects.

Classification and Sources of MPL

PL can be divided into three classes: glycerophospholipids, ether glycerolipids and sphingophospholipids. Glycerophospholipids represent the most widespread phospholipid class and they differ in their polar head groups. For example, phosphatidylcholine (PC) has choline as a head group, while phosphatidylethanolamine (PE) has ethanolamine as a head group, etc. as shown in Fig. 1. In addition, PL from different sources also have different fatty acid profiles in the *sn*-1 and *sn*-2 positions (Fig. 2a). Thereby, the chain length and degree of unsaturation may vary from source to source. For example, PL originating from plants such as soy bean do not have fatty acid chain lengths longer than 18 carbon atoms and contain only one to three double

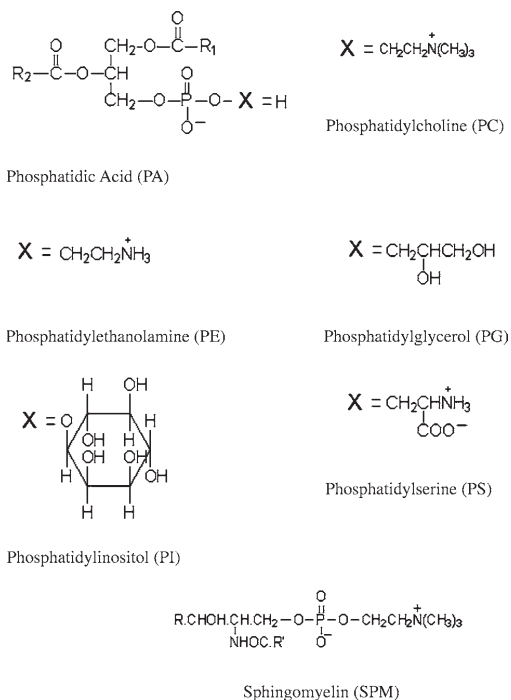


Fig. 1 Chemical structures of PL compounds with names and abbreviations

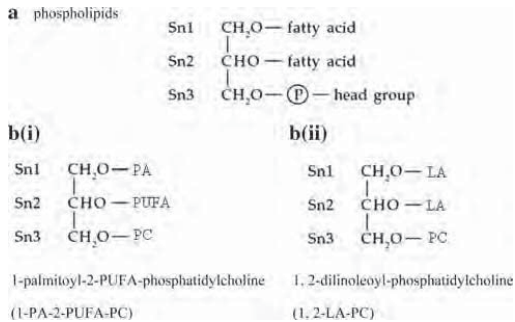


Fig. 2 **a** General structure of a phospholipid, **b i** 1-palmitoyl-2-PUFA-phosphatidylcholine **ii** 1,2-dilinoleoyl-phosphatidylcholine.

bonds, while PL originating from egg yolk or marine sources additionally have chain lengths of 20 and 22 carbon atoms with four to six double bonds e.g. as found in fatty acids of EPA and DHA. However, egg yolk only contains small amounts of EPA and DHA while marine sources are high in EPA and DHA. As far as marine sources are concerned, PL are found relatively abundant in roe, fish heads and offal such as viscera [30]. The most predominant PL in marine source such as salmon, tuna, rainbow trout and blue mackerel is phosphatidylcholine (PC) as shown in Table 1. The second most abundant is phosphatidylethanolamine (PE). Phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SPM) and lysophosphatidylcholine (LPC) are usually found in smaller amounts in marine sources, except for the relatively high level of sphingomyelin (SPM) found in tuna species [31–36]. Furthermore, krill such as *Euphausia superba* and *Euphausia pacifica* are other rich source of MPL [37, 38]. Almost half the lipid content of both types of krill is present in phospholipid form, mainly around 35% PC and 16% PE in *Euphausia superba* and 29% PC and 26% PE in

Euphausia pacifica, respectively. Currently, Neptune Krill oil (a concentrate of MPL from *Euphausia superba*) is a leading commercial krill oil on the market.

Similar to the production of egg yolk PL, production of MPL in industry uses a combination of organic solvents such as hexane and acetone, isopropanol and ethanol for extraction of wet or dried biomass [36]. Non-polar solvents are used to extract TAG while polar solvents are used to extract PL. However, extraction of lipids using organic solvents may bring adverse health effects. Recently, a more promising method without using an organic solvent, supercritical fluid extraction (SFE) has been used for the extraction and fractionation of lipids [39–42]. The extraction can be carried out at low temperature by using CO₂. However, CO₂ can only extract neutral lipids from lipid mixtures, and a generally recognized as safe (GRAS) co-solvent such as ethanol must also be used to extract PL for the food industry. For instance, the addition of about 5–10% of ethanol to CO₂ is necessary to achieve the extraction of PL from egg yolk [42–44]. Additionally, krill oil has been extracted by a patented cold vacuum extraction process that can protect the biomass from exposure to heat, light or oxygen. Thereby, the oil is protected throughout the production process and the original nutrients of the krill are maintained intact.

Health Benefits of MPL

Many studies have shown that MPL are more efficient carriers of n-3 PUFA than TAG (normal fish oils) in terms of n-3 PUFA absorption in different tissues [45–47]. Thus, MPL not only contains more n-3 PUFA than TAG from the same source [31, 48, 49], but also provide better absorption in most tissues. This may be due to the amphiphilic properties of PL resulting in better water dispersability and

Table 1 Phospholipid composition (%) of marine sources

PL classes	Salmon head lipids	Rainbow trout fillet lipids	Bigeye muscle lipids	Bluefin muscle lipids	Bonito muscle lipids	Frigate muscle lipids	Skipjack muscle lipids	Yellowfin muscle lipids	Krill	Salmon roe
PC	54.7	53.6	42.1	42.2	53.9	47.4	51.5	37.9	87.5	86.0
PE	14.0	22.9	18.8	18.9	20.1	21.8	20.2	21.0	6.3	6.0
PI	2.5	8.3	5.8	6.7	2.3	10.9	4.9	8.5	0.5	2.0
PS	10.4	4.1	5.4	4.8	2.2	5.1	5.0	5.4	0.5	ND
SPM	8.3	4.9	3.3	5.6	7.6	3.0	0.5	4.0	1.3	2.0
LPC	1.4	ND	22.1	15.4	13.8	12.0	18.3	21.5	ND	2.0
Cardiolipin	ND	6.2	ND	ND	ND	ND	ND	ND	ND	ND
Other	ND	ND	4.4	6.6	Trace	1.7	1.5	2.8	3.9	1.0

Data compiled from references [5, 31–36]

PC phosphatidylcholine, PE phosphatidylethanolamine, PI phosphatidylinositol, PS phosphatidylserine, SPM sphingomyelin, LPC lysophosphatidylcholine, ND not determined

their greater reactivity towards phospholipases compared to the glycerolysis of triglycerides [49]. For this reason, supplementation of foods with n-3 PUFA rich PL has recently emerged as an interesting way of increasing the assimilation and thereby the health benefits of EPA and DHA. EPA and DHA have numerous well-documented health benefits, which have been reviewed extensively by Narayan et al. [50]. The more recent studies on these health benefits include a reduction of coronary heart diseases, inflammation, autoimmune diseases, hypertension, cancer, diabetes, susceptibility to mental illness and neurological diseases such as depression and Alzheimer's disease, as well as improved brain and eye functions in infants [51–59].

Apart from the benefits obtained from their favorable fatty acid composition, MPL may also provide health benefits due to their polar head groups [60, 61] or to a unique combination of the two in the same molecules. The latter explanation is supported by the following observations; the use of n-3 fatty acids (EPA and DHA) in PL form (either from marine or synthetic origin), instead of the triglyceride form, together with a vegetable oil containing n-6 fatty acids in a nutritive lipid emulsion, gave even lower blood triglyceride and cholesterol levels of patients as compared to the same amount of n-3 fatty acids given as fish oil [62]. The same observation was also obtained by Bunea et al. [63] who investigated the effect of krill oil (mainly present as PL) on hyperlipidemia. In addition, they reported that high doses of krill oil significantly reduced low-density lipoproteins (LDL) level and increased high-density lipoproteins (HDL). Their study concluded that krill oil was more effective at improving blood lipids and lipoproteins than fish oil. Apart from that, several studies have also shown that krill oil has many beneficial health effects such as it may have therapeutic value for metabolic syndrome, non-alcoholic fatty liver disease, attention deficit/hyperactivity deficit disorder (AD/HD), premenstrual syndrome (PMS) and it also showed anti-inflammatory effect [64–68]. Sampalis et al. [67] reported that phospholipid krill oil was more effective than triglyceride fish oil at improving both the physical and emotional symptoms of PMS while Deutsch [66] reported that the intake of krill oil at a daily dose of 300 mg can significantly inhibit inflammation and reduce arthritic symptoms within a short treatment period of 7 and 14 days. According to Maki et al. [64], 4 weeks of krill oil supplementation increased plasma EPA and DHA of overweight and obese men and women and was well tolerated without adverse effects on safety parameters. Besides that, Hayashi et al. [69] also showed that n-3 PUFA from salmon roe phosphatidylcholine may be beneficial in treatment of chronic liver diseases while Taylor et al. [70] showed that MPL is a promising new dietary approach to tumor-associated

weight loss. Due to these numerous health benefits, there is an increasing desire to offer MPL containing n-3 PUFA to a wider market, e.g. for human foods and also to the general feed and aquaculture industry.

Introduction to Liposomes

Liposomes or lipid vesicles are aggregates formed from aqueous dispersions of amphiphilic molecules such as polar lipids that tend to produce bilayer structures [71]. They are useful microscopic carriers for nutrients and have a great potential for applications in both food and aquaculture industries. Besides that, liposomes have been recognized as a powerful tool in the treatment of diseases by the pharmaceutical industry. Their use as drug delivery vesicles and their medical applications such as in anti-cancer therapy, vaccination, gene therapy, and diagnostics have been reported in literature [72]. According to Watwe et al. [73], liposomes can be divided into three main classes: (a) multilamellar vesicles (MLV), contain more than a single bilayer membrane with a size range of 0.1–6.0 μm , (b) small unilamellar vesicles (SUV) and (c) large unilamellar vesicles (LUV) which both contain only a single bilayer membrane with sizes range of 0.02–0.05 μm and >0.06 μm , respectively. LUV are the most useful liposomes because they are more homogeneous than MLV and have higher encapsulation efficiency [74]. MPL or MPL based liposomes have obtained considerable attention and their oxidative stability has been studied extensively as shown in Table 2. Generally, MPL have been found to have a higher oxidative stability than TAG as will be discussed in the following.

Oxidative Stability of MPL

Mechanism of Oxidation for MPL

The PUFA chains in PL are the primary targets of oxidation. Similar to the oxidation of TAG, phospholipid oxidation may occur through radical and non-radical reactions involving enzymes such as lipoxygenase and myeloperoxidase or non-enzymatic systems such as $\cdot\text{OOH}$, $\cdot\text{OH}$, Fe^{2+} , Cu^+ and radiation [75]. Due to the low dissociation energy of bisallylic carbon–hydrogen in double bonds of PUFA, a hydrogen atom can easily be removed. The first steps in the lipid peroxidation consist of hydrogen abstraction, rearrangement of double bonds and addition of triplet oxygen leading to highly reactive peroxy radicals. These radicals can undergo a large variety of consecutive reactions including further reaction with other PL, fragmentation and generation of truncated PL and different

Table 2 Chemical and physical stability of MPL and MPL-based liposomes

Sources of phospholipids (PL)	Brief summary of findings	References
TL, NL and PL (from muscle of blue fish)	Antioxidant activity in salmon oil system supplemented with: 2.5% or 5% PL > 0.02% BHT 5% PL > 5% TL or 5% NL	King et al. [87]
Lipid fractions (from muscle, viscera and skin of sardine and mackerel fish)	Oxidative stability of lipid fractions: Muscle > viscera and skin Presence of higher PL (PE and PC) and α -Toc in muscle and synergistic effect of PE with α -Toc	Ohshima et al. [105]
Salmon roe PC, soybean PC	Oxidative stability of both PC in aqueous solution 1) Catalyzed by Fe ²⁺ -ascorbic acid; salmon roe PC > soybean PC 2) Under influence of emulsifier: egg albumin > Tween 20 > deoxycholic acid sodium salt Reason: high stability of salmon roe PC is due to the conformation of PC molecule and the phase behavior of PC aggregation	Miyashita et al. [90]
Squid: muscle TL, viscera TL, eye TL; Tuna orbital TL, trout egg TL and bonito TAG	Oxidative stability of lipids fraction: Squid viscera TL or squid muscle TL > squid eye TL > trout egg TL > bonito TAG > tuna orbital TL Reason: higher stability is due to the presence of PL in squid tissue lipids and trout egg TL	Cho et al. [21]
DHA, PC, PE, TG	Oxidative stability of DHA in lipids: 1-DHA-2-palmitoyl-PE or 1-palmitoyl-2-DHA-PE or 1-DHA-2-palmitoyl-PC or 1-palmitoyl-2-DHA-PC > DHA + 1,2-palmitoyl-PC (1:1) > 1,2-diDHA-PC + 1,2-dipalmitoyl-PC (1: 1) or 1,2,3-triDHA-TAG DHA was most protected against oxidation when it was incorporated at one position of either PC or PE	Lyberg et al. [9]
Fish roes: salmon and herring, commercial fish oils: crude tuna oil and sardine oil	Oxidative stability of lipids Herring roe lipids > salmon roe lipids > commercial fish oils The higher oxidative stability is mainly due to the presence of PL in fish roe lipids and the synergistic effect of PL on the antioxidant activity of α -tocopherol	Moriya et al. [25]
Salmon roe PC, chicken egg PC and commercial soybean PC	Oxidative stability of PC in: a) Aqueous micelles: Salmon roe PC > chicken egg PC > soybean PC b) Liposomes: Chicken egg PC and salmon roe PC > soybean PC Reason: Higher stability is due to the presence of PUFAs in chicken egg PC and salmon roe PC which are esterified at the <i>sn</i> -2 position	Nara et al. [6]
Salmon roe PC, chicken egg PC and commercial soybean PC	Oxidative stability of liposomes containing DHA enriched TAG: Salmon roe PC > chicken egg PC and commercial soybean PC Addition of CHO; DP, SA, chicken egg albumin and Toc improved oxidative stability of salmon roe PC liposomes	Nara et al. [7]
68% PC, 23% PE, 2% PI, 2% PS and 1% SPM, 27% CHO and 4% TAG	Low pH led to an instantaneous vesicle aggregation of MPL-liposomes and shortened the release time of vitamin B1	Cansell et al. [20]
68% PC, 23% PE, 14% EPA, 31% DHA	MPL-liposomes exhibited relative high membrane physical and chemical stability in the gastric digestion condition indicating that MPL-liposomes could be used as oral administration vectors	Nacka et al. [28]
68% PC, 23% PE, 2% PI, 2% PS and 1% SPM	Acidification caused liposomes size and shape changes while maintaining the bilayer structure indicating that MPL-liposomes could be used as oral administration vectors	Nacka et al. [27]
68% PC, 23% PE, 14% EPA, 31% DHA	α -Toc uptake after oral delivery: MPL liposomes > sardine oil digestion Under gastrointestinal condition, α -Toc incorporation improved chemical stability of liposome suspension with best oxidative stability at (5 mol%)	Nacka et al. [26]

Table 2 continued

Sources of phospholipids (PL)	Brief summary of findings	References
Cod roe PL	Lipids oxidation is proportional to $[\text{Fe}^{2+}]$ and [PL] but was dependent on pH with a maximum between pH 4 and 5 Addition of salt decreased the rate of lipid oxidation	Mozuraityte et al. [22]
Cod roe PL	Cations did not influence the rate of oxidation in ionic strength 0–0.14 M. Phosphate was more effective in reducing the oxidation rate than chloride. Salts and pH affected the zeta potential of the liposomes	Mozuraityte et al. [23]

TL total lipids, *NL* neutral lipids, *PL* phospholipids, *PC* phosphatidylcholine, *TAG* triacylglycerols, *PE* phosphatidylethanolamine, *CHO* cholesterol, *DP* diacetyl phosphate, *SA* stearylamine, *TOC* tocopherol

types of low molecular weight compounds such as aldehydes and ketones. However, enzymatic oxidation of PL can be eliminated in the MPL during their thermal production. Besides that, different PL oxidation products can be formed depending on the predominating oxidative process [76]. Oxidation products can be classified into three main categories such as: (1) long chain products that preserve the PL skeleton, and which may result from insertion of oxygen followed by rearrangement or cleavage of the PL hydroperoxides leading to epoxy, polyhydroxy, hydroxy, or keto derivatives of PL, (2) short-chain or truncated products, formed by cleavage of the unsaturated fatty acids. These products include ketones, aldehydes, unsaturated carboxylic acids, (keto)hydroxyl-aldehydes, (keto)hydroxyl-carboxylic acids, lyso-phospholipids and lyso-phospholipid halohydrins, and (3) adducts, formed by reaction between oxidation products and molecules containing nucleophilic groups, this include the products usually formed by cross-linking reactions between PL oxidation products with carbonyl groups and amino groups present in neighboring biomolecules such as peptides, proteins and phosphatidylethanolamine.

Dangers of Auto-Oxidation of MPL

Oxidation of MPL can not only deteriorate the quality of MPL enriched foods and affect the flavor, but also promote the development of neurodegenerative diseases. Many reported studies [75, 77–83] have shown that oxidized PL cause harmful effects to human health as they play pathophysiological roles in developing diseases such as age-related and chronic diseases, acute lung injury, atherosclerosis, inflammation and decrease immune response. PL oxidation products such as hydroperoxyl, hydroxyl, aldehyde and epoxy groups that are potentially important in the progression of atherosclerosis and inflammation [80]. For instance, by activating the receptor for the platelet-activating factor (PAF), oxidized PL induce platelet aggregation [84–86]. Oxidized PL can also induce monocyte adhesion to endothelial cells, accumulate in atherosclerotic lesions, and play a role in inflammation and

signaling inflammatory response. The dangers of the oxidized PL have been reviewed extensively and will not be further discussed in this review.

Antioxidant Effect of PL

King et al. [87] investigated the role of PL and the degree of fatty acid unsaturation on lipid oxidation in a salmon oil model system. Their findings showed that addition of a 2.5% (wt/wt) or a 5% (wt/wt) PL fraction extracted from bluefish to salmon oil increased its stability during heating at 55 and 180 °C as compared to the control salmon oil, or salmon oil to which 0.02% (wt/wt) of BHT or 5% (wt/wt) of other lipid fractions from bluefish such as total lipid or neutral lipid had been added. The PL fraction with 34% DHA was found to exhibit higher oxidative stability than other lipid fractions with 15% DHA. Subsequently, they investigated the antioxidant properties of individual PL in a salmon oil model system [88]. They found that nitrogen-containing PL such as PE, PC, LPC, and SPM were equally effective as antioxidants and they were more effective than PS, PG and PI. Their studies did not postulate any mechanism or reasons for the antioxidant properties of the different PL classes. In both studies by King and colleagues, the oxidative stability of the salmon oil model system was investigated through 2-thiobarbituric acids (TBARS) assay and the decreases in the ratio of DHA to PA (C22:6/C16:0). Boyd et al. [89] investigated the effect of 0.5% (by weight) PL toward lipid oxidation of 2.5 g salmon oil and menhaden oil model systems respectively, through the more sensitive headspace gas chromatographic analysis. Their study also showed that addition of PL significantly reduced the production of volatile compounds in both oil model systems.

Conformations of PUFA at the *sn*-2 Position of PL

Miyashita et al. [90] showed that salmon roe PC had a higher oxidative stability than soybean PC in an aqueous solution dispersed with chicken egg albumin although the degree of unsaturation in the salmon roe PC was higher

than in the soybean PC. They suggested that the high stability of salmon roe PC was mainly correlated with the conformation of the PC molecule and the phase behavior of PC aggregation. The main molecular species of soybean PC was 1,2-dilinoleoyl-phosphatidylcholine (1,2-diLA-PC), while for salmon roe PC it was 1-palmitoyl-2-PUFA-phosphatidylcholine (1-PA-2-PUFA-PC) as shown in Fig. 2b. Hence, the presence of this main molecular species in salmon roe PC (with most of the PUFA located at the *sn*-2 position of PC) may provide a more tightly packed molecular conformation as compared to the soybean PC and thereby increase resistance of PC towards oxidation. The findings of Miyashita et al. [90] corroborated the original work of Applegate and Glomset [91] who reported that DHA in the *sn*-2 position of diacylglycerol (DAG) containing a saturated acyl chain in the *sn*-1 position could form a tighter intermolecular packing conformation as will be further discussed below.

Conformations of DHA at the *sn*-2 Position in a DAG Model

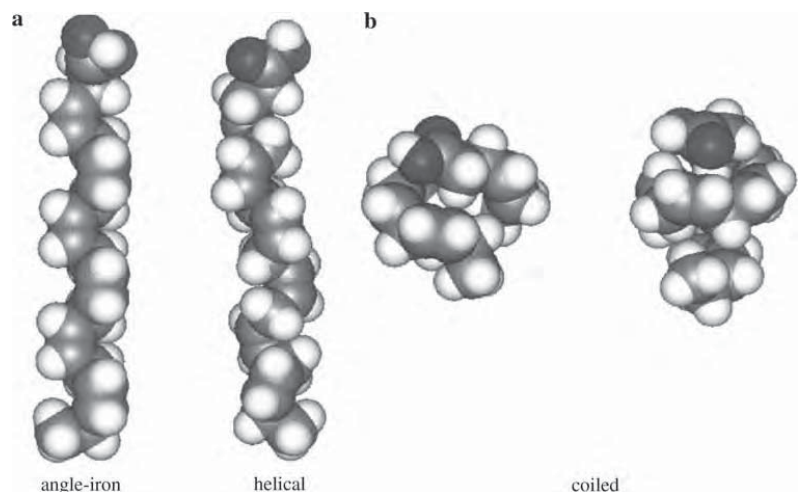
Applegate and Glomset (1986) used a molecular modeling approach to search for conformations of DHA that might uniquely influence acyl chain packing in cell membranes. Their DHA conformations of lowest energy as shown in Fig. 3 were extended conformations in which six double bonds projected outward from the methylene axis (a) in two nearly perpendicular planes to form an extend angle-iron shaped structure or (b) at nearly 90° intervals to form a helical structure, respectively. Studies of packed arrays of these hexaenes with or without saturated hydrocarbons showed that tight packing arrangements were possible

especially for angle iron-shaped molecules as a consequence of back-to-back, intermolecular contacts involving these chains. Applegate and Glomset [92, 93] further concluded that different unsaturated fatty acids at the *sn*-2 position of *sn*-1,2-diacylglycerols (DAG) may promote different packing and conformations. For instance, 1-stearoyl-2-DHA-DAG and 1-stearoyl-2-AA-DAG can assume a regular shape and tight packing while 1-stearoyl-2-oleoyl-DAG adopt a highly irregular shape and much looser packing. The simulations by Applegate and Glomset were done without reference to potential effects of polar headgroups, water of hydration and applied thermal energy. However, the molecular areas obtained for the model of DAG are in good agreement with that of the *sn*-2 polyunsaturated phosphoglycerides [94, 95]. This raises the possibility that corresponding natural phosphoglycerides may be able to pack closely together in monolayers and bilayers if their headgroups do not interfere. The findings of Applegate and Glomset were supported by Albrand et al. [96] who also agreed with the existence of the extended-helical conformations of DHA in PL. However, they also suggested several coiled conformations for DHA, tightly back-folded helical conformations with 1.2 and 1.5 spirals appearing to be the most stable as shown in Fig. 3.

More Recent Studies on the Conformation of PUFA at the *sn*-2 Position of PL

Nara et al. [6, 7] further compared the oxidative stability of PC from salmon roe, soybean and chicken egg in aqueous micelles and also in the form of liposomes with and without encapsulation of lipophilic substances. In aqueous

Fig. 3 Extended conformations of DHA in (a) angle-iron shaped and helical form, (b) coiled form



micelles, salmon roe PC was found to have the highest oxidative stability as evaluated by the highest content of un-oxidized PUFA, followed by chicken egg PC and soybean PC. Their findings are in agreement with the findings of Miyashita et al. [90]. No significant difference was found in oxidative stability between chicken egg PC and salmon roe PC when in the pure form of liposomes. However, for liposomes encapsulating with DHA enriched TAG resulted in the highest oxidative stability of both TAG and PC when salmon roe PC was used as the encapsulation material [7]. This unusual order of oxidative stability could be expected to be closely related to the conformation of PUFA at the *sn*-2 position in PC molecules as mentioned earlier [91]. Consequently, it is difficult for free radicals and oxygen to attack PUFA in bilayers of tighter conformation in salmon roe PC liposomes. Nara et al. [7] also suggested the possibility of using salmon egg PC as a liposomal material for the prevention of the oxidation of encapsulated fish oils.

Furthermore, Araseki et al. [8] also reported the characteristic oxidative stability of PC liposomes prepared from synthesized PC containing palmitic acid (PA), linoleic acid (LA), arachidonic acid (AA) and docosahexaenoic acid (DHA) in known positions. When the oxidative stability of 1-PA-2-LA-PC or 1-PA-2-AA-PC was compared with that of a 1:1 (mol ratio) mixture of 1,2-diPA-PC + 1,2-diLA-PC, or 1,2-diPA-PC + 1,2-diAA-PC respectively, the PC were more oxidatively stable than the latter corresponding PC mixtures in all oxidation systems despite the fact that the degree of unsaturation was the same in 1-PA-2-PUFA-PC and the corresponding mixture of PC. This was suggested to be due to the different conformation of PC bilayers which refer to the location of PUFA at the *sn*-2 position and the different rate of hydrogen abstraction by free radicals from intermolecular and intramolecular acyl groups. Their finding did not support a study by Lyberg et al. [9] who reported that the stability of DHA was improved independent of its position (*sn*-1 or *sn*-2) in PC or PE. Besides that, the more recent experiments and simulations [97–102] emphasized various degrees of flexibility of the DHA chain that gives looser packing of lipids bilayer. Their NMR analysis showed that the mobility of the hydrophobic part of the DHA molecule is higher than that of LA in liposome formation. These two competing views were portrayed in a review by Gawrish et al. [103]. However, according to Saiz and Klein [100], the flexibility of DHA chain conformation gives looser packing of the membrane at the lipid water interface and causes high water permeability. The presence of water molecules near DHA molecules lowers the density of the bisallylic hydrogen and inhibits the hydrogen abstraction from double bonds of PUFA during the propagation stage of auto-oxidation. As a conclusion, the higher water permeability

of DHA and its specific conformation may be a reason for higher oxidative stability of DHA or other PUFA containing liposomes.

However, as compared to the study mentioned earlier by Miyashita et al. [90], contradictory results have also been reported by Monroig et al. [15, 16, 19] in their efforts to develop PUFA-rich liposomes for fish feed. They found that liposomes made from krill PL with 67% PC, 9% PE and a high content of PUFA showed lower oxidative stability as compared to liposomes made from soybean lecithin with 95% PC. The contradictory findings may be due to the different experimental conditions in the two studies, liposomes in model system versus liposomes in *Artemia* enrichment condition. In the model system, liposomes were formulated with pure PC containing fatty acid chains in known positions of the glycerol moiety and the oxidation was carried out in a very well-defined condition (temperature of 37 °C, in the dark and without agitation). On the contrary, the *Artemia* enrichment conditions were as follows: enrichment was carried out at 28 °C with strong aeration and 21 h of incubation.

Synergism Between PL and α -Tocopherol

Many studies have shown that the higher stability of PL may be due to the presence of antioxidants such as α -tocopherol in the PL mixture or synergistic effects of PL together with α -tocopherol [21, 25, 87, 88, 104–107]. The mechanism responsible for the synergy of tocopherols and PL is not very well understood. However, Hildebrand et al. [108] postulated that the mechanism involved in synergism of PE, PC and PI with tocopherol in the autoxidation of soybean oils were as follows: (1) amino groups of organic bases in PE and PC molecules and reducing sugar in the PI molecule facilitate hydrogen or electron donation to tocopherol and (2) these PL extend the antioxidant efficacy of tocopherol by delaying the irreversible oxidation of tocopherol to tocopherylquinone. Additionally, Saito et al. [106] reported that antioxidant activity of PL was found to be attributable not only to side chain amino groups such as choline and ethanolamine, but also to the hydroxyl group in the side chain.

Oshima et al. [105] studied the oxidative stability of sardine and mackerel lipids with respect to synergism between phospholipids and α -tocopherol. They investigated the oxidative stability of lipid fractions from different parts of sardine and mackerel; tissue from white and red muscles, viscera and skin of the fish. The oxidative stability was determined through the measured changes of the peroxide value (PV), fatty acid composition, α -tocopherol content and the oxygen uptake of lipids during an incubation period at 37 °C. Muscle lipids, which contain α -tocopherol and larger amounts of PL (PE and PC) than

other tissues, showed good oxidative stability despite their high content of PUFA. It was postulated that the synergistic effect of PE with α -tocopherol was the main reason for this phenomenon. Cho et al. [21] compared the oxidative stability of lipid fractions from marine organisms, squid muscle total lipids (TL), squid viscera TL, squid eye TL, tuna orbital TL, trout egg TL and bonito TAG. The fatty acid compositions, lipid classes, tocopherol contents and average number of bisallylic positions in each lipid fraction are shown in Table 3. Higher oxidative stabilities of three kinds of squid tissue TL and trout egg TL compared to those of bonito TAG and tuna orbital TL were observed as shown in Fig. 4. The authors suggested that the presence of PL in lipid fractions from squid tissue and trout egg was responsible for this increased oxidative stability. In addition, bonito TAG was found to be less susceptible to oxidation than tuna orbital TL and this could be due to the presence of a higher tocopherol content in bonito TAG.

Moriya et al. [25] compared the oxidative stability of fish roe lipids (salmon roe and herring roe) with that of lipids

from commercial fish oils (crude tuna oil and crude sardine oil). As shown in Table 4, fish roe lipids contain higher levels of PL, EPA and DHA, and lower levels of tocopherol while lipids from commercial fish oils contain higher levels of TAG, tocopherol and lower EPA and DHA levels. Judging from these data, fish roe lipids were presumed to have lower oxidative stability. However, the opposite was observed as shown in Fig. 5 and it was proposed that the higher oxidative stability of fish roe lipids was mainly due to their high content of PL. It was also suggested that the synergistic effect of PL on the antioxidant activity of tocopherol was the main reason for this phenomenon. The higher oxidative stability of herring roe as compared to salmon roe was suggested to be due to synergism between PE and tocopherol. As shown in Table 4, the PE content in herring roe lipids was 6.6%, but there was no PE in salmon roe. Furthermore, herring roe also contained higher levels of PS and lysoPC than salmon roe and this may also have caused differences in their oxidative stability. The presence of antioxidants other than tocopherols in fish roe lipids such

Table 3 Composition of lipids from marine sources

Fatty acids (wt%)	Squid muscle TL	Squid viscera TL	Squid eye TL	Tuna orbital TL	Trout egg TL	Bonito TAG
14:0	2.1	4.4	0.9	2.9	3.6	3.3
16:0	32.7	15.9	23.2	17.0	10.7	16.3
18:0	4.4	2.9	5.6	3.0	3.0	4.1
18:1n-7	1.3	3.1	1.6	2.9	3.3	2.4
18:1n-9	1.3	8.7	0.2	23.8	15.8	13.8
20:1n-7	ND	2.8	ND	ND	1.7	ND
20:1n-9	2.5	4.2	3.4	1.8	1.8	0.9
18:2n-6	0.2	1.3	1.4	ND	1.1	3.6
18:3n-3	0.1	ND	0.2	ND	1.5	ND
20:3n-3	ND	ND	4.8	0.5	2.7	ND
20:4n-6	1.9	1.7	ND	2.0	0.7	ND
20:5n-3	10.6	12.3	15.1	4.8	18.4	0.6
22:6n-3	38.1	22.5	37.7	21.0	19.8	26.1
No. of bisallylic positions ^a	2.51	2.11	2.77	1.65	2.19	1.92
Lipid class (% of total lipids)						
Triacylglycerols	ND	95.5	ND	99.3	76.8	99.6
Free fatty acids	ND	ND	ND	0.4	ND	0.1
Glycolipids	ND	ND	6.8	ND	ND	ND
Sterols	23.7	0.7	28.3	ND	2.2	0.3
Phospholipids	75.6	3.8	66.4	0.2	23.1	ND
Tocopherol content ($\mu\text{g g}^{-1}$ lipid)						
α -tocopherol	649.8	212.5	1198.8	541.3	215.5	253.4
β -tocopherol	ND	ND	ND	ND	ND	193.3
γ -tocopherol	ND	ND	ND	ND	ND	703.6
δ -tocopherol	ND	ND	9.2	ND	9.2	496.3
Total tocopherol	649.8	212.5	1208.0	541.3	215.5	1646.6

Data from reference [21]

ND not detected

^a Per one fatty acid molecule

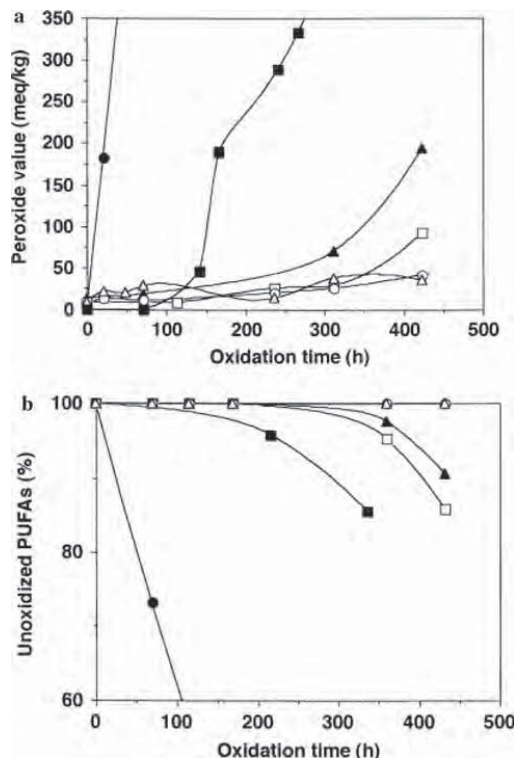


Fig. 4 **a** Changes in the peroxide value (PV) and **b** unoxidized PUFA in lipids from marine organisms during auto-oxidation at 37 °C. (*open triangle*) Squid viscera total lipids (TL); (*open circle*) squid muscle TL; (*open square*) squid eye TL; (*filled circle*) tuna orbital TL; (*filled triangle*) trout egg TL; (*filled square*) bonito oil. Reproduced from Cho et al. [18] with permission from John Wiley & Sons Ltd

as astaxanthin, coenzyme Q10 and lutein might contribute to this extraordinary stability as well.

Other studies [104, 107, 109] reported that the synergistic effect of PE with α -tocopherol was higher than that of PC. Bandarra et al. [104] investigated the antioxidant synergy of α -tocopherol (0.04%) with several PL fractions (0.5%) such as PE, PC and cardiolipin (CL) in a refined sardine oil model system. Their results showed that PC was the most effective individual antioxidant when it was compared to PE, CL and α -tocopherol while PE provided the highest synergistic effect with α -tocopherol. Higher synergism of PE as compared with that of PC could be due to the easier hydrogen transfer from the amino group of PE to tocopheroxyl radical and regeneration of tocopherol or the secondary antioxidant action of PE in reducing quinones formed during oxidation of tocopherols [109]. Since MPL may contribute to better oxidative stability than marine TAG, it can be expected that enrichment of foods or

food emulsions with MPL could lead to n-3 PUFA enriched foods that have better oxidative stability than foods enriched with n-3 TAG.

Stability of MPL Based Liposomes Under Gastrointestinal Conditions

MPL based liposomes were designed with the purpose of increasing the PUFA bioavailability and also to protect entrapped compounds from digestive degradation. However, liposome characterization with respect to vesicle composition and membrane integrity under various gastrointestinal conditions are needed before considering liposomes as a useful oral dosage form. Many studies have shown that MPL liposomes could be used as an oral administration vector [6, 7, 20, 26–28]. This is because bilayer structures of MPL based liposomes were still maintained even under acid stress or gastrointestinal conditions despite of slight morphological modifications. Nacka et al. [28] investigated the in vitro behavior of MPL based liposomes under the influence of pH from 1.5–2.5 (stomach) to 7.4 (intestine) at physiological temperature (37 °C) in the presence of bile salts and phospholipase A₂ (Table 2). Their study showed that acidification induced instantaneous vesicle aggregation of MPL based-liposomes, which was partially reversed when the external medium was neutralized. Acidification also caused a complex morphological bilayer rearrangement and led to the formation of small aggregates. Nevertheless, Nacka et al. [27, 28] reported that the pH and temperature dependent structural rearrangement is mainly due to the osmotic shock and chemical lipid alterations such as oxidation and hydrolysis. Hydrolysis of the liposomes was amplified under the influence of an acid medium and high temperatures (Table 2).

Cansell et al. [20] investigated the physical stability of MPL-based liposomes containing vitamin B1 under acidic conditions simulating the stomach conditions. Encapsulation of vitamin B1 in the liposomes was carried out through passive encapsulation and active loading methods. They observed that vitamin B1 was totally released from liposomes after 24 h storage in a neutral medium and the time of release was shortened to 1 h in acidic condition (pH 1.5). According to their study, this liposome instability could result from the external medium osmolarity that forced water to flow out of the liposomes and simultaneously dragged vitamin B1 molecules through the bilayer. Furthermore, protons may also destabilize the lipid membrane by their interaction with PL via structural membrane rearrangement as previously mentioned. However, their study also proved that addition of xanthan gum improved the encapsulation efficiency and also the retention of vitamin B1 in liposomes regardless of the encapsulation

Table 4 Composition of marine lipids used for oxidation

Lipid class (% of total lipids)	Crude tuna oil	Crude sardine oil	Salmon roe	Herring roe
Triacylglycerols	99.6	99.8	71.8	9.3
Free fatty acids	0.1	0.2	ND	3.8
Phospholipids	ND	ND	23.1	73.6
Sterols + monoacylglycerols	0.3	ND	7.2	12.3
% of phospholipids				
PC	ND	ND	97.0	72.3
PE	ND	ND	ND	6.6
PS	ND	ND	2.6	8.7
LysoPC	ND	ND	ND	11.8
Fatty acid profiles				
14:0	3.3	4.1	3.6	2.1
16:0	16.3	8.0	10.7	25.8
18:0	4.1	1.4	3.0	2.2
18:1n-7	2.4	2.0	3.3	5.1
18:1n-9	13.8	10.9	15.8	13.2
20:4n-6	–	1.3	0.7	1.0
20:5n-3 (EPA)	0.6	21.8	18.4	14.4
22:6n-3 (DHA)	26.1	13.7	19.8	21.6
EPA + DHA	26.7	35.5	38.2	36.0
Tocopherol content ($\mu\text{g g}^{-1}$ lipid)				
α -tocopherol	253.4	60.2	19.6	22.9
β -tocopherol	193.3	45.7	214.1	258.0
γ -tocopherol	703.6	376.7	11.6	7.7
δ -tocopherol	496.3	2670.9	11.3	11.5
Total tocopherol	1472.6	3153.5	256.6	300.1
Other antioxidants ($\mu\text{g g}^{-1}$ lipid)				
Astaxanthin	ND	ND	156	ND
Coenzyme Q10	ND	ND	24	100
Lutein	ND	ND	ND	6.4

Data from reference [25]

ND not determined, *LysoPC* lysophosphatidylcholine, *PE* phosphatidylethanolamine, *PL* phospholipids, *PS* phosphatidylserine

method used. They suggested that this increase is due to the adsorption of hydrocolloid to the outer surface of the liposomes that not only trapped part of the external vitamin but also formed a strong xanthan gum coating around the liposome surface. They postulated that this coating resulted from strong lipid–hydrocolloid interactions occurring during the centrifugation steps of liposome preparation.

The Effect of Lamellarity, pH, Temperature, Ionic Strength, Presence of Pro-oxidants and Chelators on MPL-Based Liposomes' Stability

Chemical and physical stability of liposomes are closely related to the mechanical strength and lipid bilayer conformation. Strong and well-packed lipid bilayers or multilamellar layers can protect the entrapped substance, decrease the changes of size distribution, fusion or other changes in the mechanical properties of lipid bilayers. For this reason, factors such as lamellarity, pH, temperature,

ionic strength, dissolved oxygen content within the formulation, the presence of antioxidants and chelators are believed to affect mechanical properties of lipid bilayers and thereby affect the physical and chemical stability of MPL-based liposomal products [22, 23].

Nacka et al. [27] showed that the sensitivity of MPL based liposomes towards harsh condition such as acidic condition depends on their size and lamellarity (Table 2). They found that filtered liposomes with higher lamellarity and a protective effect against aggregation showed a slower size rearrangement. This finding supported a study by Monroig et al. [19] who, in addition, reported that liposomes with multilamellar vesicles seem to be more suitable than liposomes with unilamellar vesicles in the encapsulation of free methionine. They found that methionine dissolved in the more internal intermembrane spaces of multilamellar liposomes would remain encapsulated, whereas methionine from the aqueous compartments located between the more outer membranes would leak out

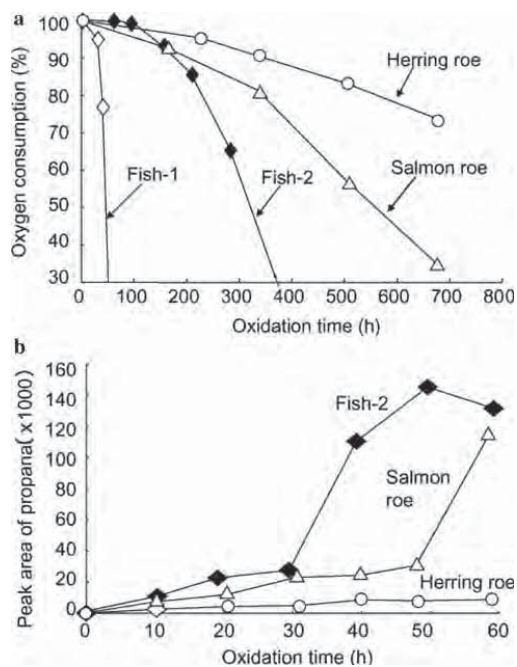


Fig. 5 **a** Oxygen consumption during the oxidation of fish lipids at 37 °C in the dark. (open diamond) fish-1; (filled diamond) fish-2; (open triangle) salmon roe lipids; (open circle) herring roe lipids. **b** Propanal formation during the oxidation of fish lipids at 37 °C in the dark. (filled diamond) fish-2; (open triangle) salmon roe lipids; (open circle) herring roe lipids. Reproduced from Moriya et al. [22] with permission from John Wiley & Sons Ltd.

into the external medium when the liposomes were subjected to harsh conditions. However, this result contradicts another study by this group [15] where unilamellar liposomes were found to be more stable than multilamellar liposomes. The apparent discrepancy in these two studies is probably due to different experimental conditions and materials used for the liposomes preparation.

Mozuraityte et al. [22] examined the lipid oxidation rate of liposomes made from cod PL under influence of factors such as the temperature, the amount of added Fe^{2+} , the lipid concentration, pH, the concentration of NaCl, and the dissolved oxygen. Their study showed that the rate of lipid oxidation was proportional to the iron and lipid concentrations. Furthermore, lipid oxidation was dependent on pH, with a maximum observed between pH 4 and 5. Addition of NaCl decreased the rate of lipid oxidation. However, contradictory results were reported in another study [110] which showed that addition of NaCl had no effect or even increased iron-catalyzed oxidation of a sodium dodecyl sulfate-stabilized salmon oil emulsion.

Mozuraityte et al. [23] examined the effect of zeta potential on the lipid oxidation rate of liposomes made from cod PL under the influence of pH and different cations such as Na^+ , K^+ , Ca^+ , Mg^+ and anions such as H_2PO_4^- and Cl^- (Table 2). Their data showed that cations did not influence the rate of oxidation in the tested range of the ionic strength from 0 to 0.14 M whereas the opposite was the case for anions. Both phosphate and chlorides have an additive antioxidative effect on the oxidation in liposomes. Phosphate was shown to be more effective in reducing the oxidation rate than chloride. The inhibition of Fe^{2+} induced oxidation of liposomes by phosphate might be due to the phosphate chelation of iron [111, 112]. Furthermore, they also concluded that addition of salts and changes in pH affected the zeta potential of the liposomes. However, absolute values of the zeta potential alone cannot be used to predict oxidation rates.

Improvement of MPL Based Liposomes' Oxidative Stability

Many studies have been conducted to improve the oxidative stability of liposomes. Most of the studies focus on the use of cholesterol in improving the oxidative stability of liposomes [12, 113–115]. For example, a study conducted by Nara et al. [7] showed that addition of cholesterol and ingredients such as diacetyl phosphate (DP) and stearylamine (SA) improved the oxidative stability of salmon roe PC liposomes. Furthermore, in the effort of developing liposomes as feed supplement in larva culture. Monroig et al. [15] also showed that addition of cholesterol to liposomes made from krill PL or 1,2-PA-PC or soy PC improved the oxidative stability of the liposomes. Cholesterol has a condensing effect on the PC bilayer arrangement over its phase transition temperature and thus improves the physical stabilization of PC liposomes [116]. Addition of cholesterol can increase the rigidity of 'fluid state' liposomal bilayers and the retention of entrapped hydrophilic substances [117]. It counteracts lipids phase transition and increases resistance to in vivo liposomes degradation [118–120]. An interaction mechanism between bilayer forming PL and cholesterol has been proposed. This is due to the formation of hydrogen bonds between the three hydroxyl group of cholesterol and fatty acyl esters of PL at both *sn*-1 and *sn*-2 positions [121, 122]. These physico-chemical effects of cholesterol on liposomes may contribute to the increased oxidative stability in liposomes with cholesterol.

α -Tocopherol is widely known for its antioxidative effect [123]. However addition of high concentrations of α -tocopherol may also cause prooxidative effects [124, 125]. The most effective concentration of α -tocopherol in the prevention of lipid oxidation in salmon roe PC liposome

suspensions was 0.25 μM in a study conducted by Nara et al. [7]. Nacka et al. [26] investigated the most efficient amount of α -tocopherol for liposomes incorporation under gastrointestinal-like conditions. Their findings showed that the best oxidative stability was obtained for liposomes that were prepared at a ratio of 5 mol% of α -tocopherol of the total marine lipids. This concentration of α -tocopherol produced liposomes with the lowest concentration of propanal as an oxidation product of n-3 PUFA and required the longest time of oxidation induction phase. They also found that incorporation of α -tocopherol induced liposome structural modifications, evidenced by turbidity and the production of lysophospholipids from PL chemical hydrolysis.

Nara et al. [6, 7] investigated the effect of addition of diacetyl phosphate (DP), stearylamine (SA) and chicken egg albumin, and soybean protein on improving the oxidative stability of MPL based-liposomes. DP and SA give a negative or positive charge to the liposomes respectively and thus protect the liposomes from aggregation. An improved oxidative stability of liposomes after addition of this ingredient was observed and suggested that it was due to the physical stabilization of the PC liposomes. Furthermore, added proteins such as chicken egg albumin and soybean protein improved the oxidative stability of liposomes by protecting the PC bilayer from the attack of free radicals. Proteins have the ability to adsorb at PC–water interfaces and this adsorption of proteins would closely relate to its antioxidant activity [6]. However, albumin acted as a more effective inhibitor of the oxidation of PC containing DHA than PC containing LA [90].

Determination of Oxidation Products from MPL

As discussed above MPL has been found to exert antioxidant effects toward lipids oxidation. However, many of the lipid oxidation studies [6–8, 21, 90, 105] were performed using simple analyses such as TBARS, PV, determination of the un-oxidized lipids (PUFAs) content through gas chromatography, or determination of only one secondary volatile compound, propanal (as a marker of n-3 PUFA oxidation) by headspace GC–MS analysis [25], etc. In many of these oxidative stability studies, there is a lack of determination of the entire spectrum of volatile oxidation products or identification of specific oxidation products which are responsible for sensory off-flavors of the marine lipids. Furthermore, there are no studies providing the sensory data or statistical correlation between instrumental analysis and sensory data for oxidation of MPL. These data are particularly important in the studies of MPL for foods enrichment and additional studies in this area are clearly needed. Due to the low odor threshold, the presence of volatile secondary oxidation products, even at low

concentrations, can significantly decrease the sensory quality of marine lipids or marine lipids containing foods.

In the recent years, the oxidation products of PL have attracted intensive research interest due to their biological functions in human pathophysiology. Similar to other lipids such as TAG, many methods can be used to study the oxidation of PUFA containing PL such as (1) measurement of lipid hydroperoxides through spectrophotometric determination of PV or conjugated dienes (CD). Lipid hydroperoxides may also be determined by sample derivatization followed by HPLC with chemiluminescence detection, (2) measurement of breakdown products of hydroperoxides, such as the aldehydes, malondialdehyde, etc. through anisidine value (AV), 2-thiobarbituric acid value (TBARS), etc., (3) measurement of secondary volatile compounds through more sensitive instrumental methods such as GC–MS, (4) measurement of long chain oxidation derivatives of PL through MS. Electrospray ionization (ESI) is gaining in popularity in this area nowadays for this purpose [76]. ESI is a soft ionization technique that does not cause fragmentation and allows detection of intact PL classes without sample derivatization. ESI can readily be coupled to reverse phase LC and allow the analysis of oxidized PL [126–129]. Interfacing reverse phase LC to ESI–MS has the advantage as oxidized PL elutes earlier than their native counterparts due to their higher hydrophilicity. Spickett et al. [127] used the positive ion ESI–MS for detection of hydroperoxide in PC vesicles after treatment with *tert*-butylhydroperoxide and Fe^{2+} while Yin et al. [129] used ultra performance liquid chromatography (UPLC) coupled with negative ion electrospray ion trap MS to identify the intact oxidation products of glycerophospholipids in vitro and in vivo such as hydroxyeicosatetraenoates (HETE) and isoprostanes (IsoP). Other soft ionization methods include matrix-assisted laser desorption ionization (MALDI) and tandem mass spectrometry (MS/MS). As a conclusion, the future direction for research and development could focus on the investigation of oxidative stability for MPL by using advanced MS analysis.

Potential of MPL as Liposomal Material

A variety of liposome preparation methods are available nowadays ranging from traditional methods using solvent extraction such as thin film hydration, detergent dialysis, reverse-phase evaporation, etc. to emerging technologies without using an organic solvent such as pro-liposome, supercritical fluid extraction, and microfluidization. Each method has its own advantages and drawbacks as reviewed by Taylor et al. [130]. Among these technologies, pro-liposome and microfluidization are recommended to produce liposomes for food applications. Pro-liposome is a

simple method for mass production of liposomes without using large amounts of energy, solvents and complex equipment. This method is based on the idea that addition of water to an appropriate mixture of ingredients leads to the spontaneous formation of liposomes [29]. On the other hand, microfluidization is a method using a microfluidizer (a high pressure homogenizer) that can rapidly produce large volumes of liposomes in a continuous and reproducible manner. The average size of the liposomes can be adjusted through this technology and the solutes to be encapsulated are not exposed to sonication, detergents or organic solvents. Furthermore, this technology enables the production of stable liposomes with high encapsulation efficiency [74]. Recently, Thompson et al. [131–133] used a microfluidization technique to produce liposomes from milk fat globule membrane PL in the food industry. Studies showed that liposomes prepared via microfluidization have high encapsulation efficiencies, smaller size, a narrower size distribution and a higher proportion of unilamellar vesicles as compared to methods such as thin film hydration. PL from soybean and egg yolk, either in purified form, crude form or hydrogenated form are widely used for liposome production in both the food and aquaculture industries. The use of MPL-based liposomes has gained attention recently in the aquaculture industry and there is much ongoing research in this area as shown in Table 5. Several studies have shown the use of MPL such as herring roe or krill PL for larvae feed in the aquaculture industry [14–19] but no attempts to use MPL based liposomes for food purposes have been reported in the literature so far.

One potential advantage of using MPL-based liposomes for food application is that they may provide better bio-availability of encapsulated nutrients [26, 134, 135] as compared to TAG. Nacka et al. [26] showed that MPL-based liposomes facilitated α -tocopherol uptake after oral delivery as compared to sardine oil digestion. Furthermore, Hossain et al. [136] also showed that MPL-based PC liposomes (squid PC and starfish PC) enhanced the permeability, transportation and uptake of PL in Caco-2-cells. It is also known that the fluidity of liposomes increases with increasing contents of highly unsaturated PUFA such as AA and DHA, showing the advantage of PC containing AA or DHA for use in drug or nutrient delivery systems [100, 101].

Application of PL Liposomes in the Food Industry

The uses of liposomes in the food industry can be summarized as follows (1) use of liposomes to encapsulate food ingredients in order to provide better protection or to hide the bitter taste of entrapped substances and (2) use of liposomes to control the delivery of functional components by

delaying the release of the encapsulated materials. Liposomes have been used to entrap thermally sensitive compounds such as vitamins, enzymes, flavorings, PUFA from fish oils, antimicrobial peptides (lysozyme, nisin) and other nutrients [13, 137–144]. Hydrophilic substances can be entrapped in the internal water core of the liposomes while lipophilic compounds can be efficiently enclosed in the PL bilayer at the same time through a pro-liposomes approach [29]. For this reason, liposomes can be used for the formulation of functional foods or drinks such as energy drinks, sport drinks, fortified milk, etc. Arnaud et al. [145] reported that PC from egg or soybean has been used in development of liposome-based functional drinks. With the use of PC-based liposomes in food industry, consumers not only benefit from the health benefits of water soluble nutrients that are entrapped in the liposomes but also benefit from the nutritional benefits of PL in liposomes. In the production of cheese, PL liposomes may be used to delay the release of encapsulated proteinases [146, 147] or to protect encapsulated enzyme such as protease and lipases with the purpose of improving the texture and sensory properties of cheese [148–152]. Liposomes have also been used to encapsulate vitamin D with the purpose of increasing the vitamin D content of cheese [153].

Application of PL Liposomes in the Aquaculture Industry

Besides food incorporation, recent studies have also indicated that liposomes rich in n-3 PUFA can offer a range of benefits when used for fish larvae feed. Due to the high consumer demand and limited natural stocks of fish species such as salmon, trout and eel, much effort has recently been spent by researchers on developing cost effective aquaculture methods for farming such species. Generally, the main problems faced by aquaculture industry are low survival rate of the hatched fish larvae of the farmed species and the difficulty in supplying live prey organisms which provide nutritionally adequate feed for these larvae. Live prey such as Rotifers *Brachionus plicatilis* and *Artemia* nauplii provide adequate amounts of protein and energy. However, they do not provide lipid profiles that cover the requirements for EPA and DHA, which are essential for optimum survival, growth and development of larvae [154–157]. Thus, to provide prey organisms with such a composition of n-3 PUFA, it is necessary to cultivate these organisms in the presence of enrichment products with high EPA and DHA contents, preferably in an easily digestible, highly bio available form, such as MPL. During the enrichment process, enrichment products are passively filtered by *Artemia* nauplii and their digestive tract becomes loaded with these enrichment products. A wide

Table 5 Application of liposomes in the aquaculture industry

Sources of liposomes	Brief summary of findings	References
Purified PC, CHO, PG, menhaden oil	It is feasible to use liposomes for <i>Artemia</i> nauplii enrichment with PL and free amino acids such as glycine, liposomes were readily ingested and assimilated by <i>Artemia</i> nauplii as indicated by ^{14}C -glycine and ^{14}C -PC	Ozkizilcik and Chu [169]
1,2-PA-PC, egg PC, bovine brain PS	It is feasible to use liposomes in aquaculture as a delivery system through <i>Artemia</i> nauplii. PUFA rich liposomes were stable at least for 3 days at room temperature without agitation and freeze drying could stabilize liposomes for long term storage	Hontoria et al. [170]
PE, CHO, Toc	It is feasible to use liposomes as a delivery system of water soluble antibiotics, oxytetracycline for marine larvae	Touraki et al. [167]
1,2-PA-PC, herring roe PC, CHO	<i>Artemia</i> nauplii enrichment with MPL emulsion or MPL-liposomes significantly increased: DHA level (% of TL) DHA:EPA ratio: Polar lipids content: PT (14%) > SS PT (1.8) > SS L (40.1 mg g ⁻¹) > PT (6.3%) > L (2%) (0.4) > L(0.3) (32.4 mg g ⁻¹) = SS (34.7 mg g ⁻¹)	McEvoy et al. [165]
Egg yolk lecithin (60%PC), CHO	Consumption rate of liposomes in gilthead seabream (<i>Sparus aurata</i>) and white grouper (<i>Epinephelus aeneus</i>) larvae: Liposomes containing CFE (238.5 ng liposome larva ⁻¹ n ⁻¹) > liposomes containing PHS (54.3 ng liposome larva ⁻¹ n ⁻¹) It is feasible to use liposomes as a nutrient supplement in first feeding marine fish larvae	Koven et al. [163]
Crude egg yolk PC (>60%)	Content of methionine in <i>Artemia</i> nauplii after different enrichment methods:	Tonheim et al. [168]
Purified egg yolk PC (> 99%), CHO	Purified egg PC liposomes > crude egg PC liposomes > direct enrichment with free methionone > unenriched control	
1,2-PA-PC, Krill PL, soy PC, CHO	Oxidative stability of formulated liposomes: (100%)Soy PC > (100%)Krill PL (40%)1,2-PA-PC (40%)Krill PL(20%)CHO > (80%)Krill PL(20%)CHO LUV > MLV Addition of CHO improved oxidative stability	Monroig et al. [15]
Krill PL (mainly PC, PE)	EFA bioencapsulation depends on methods preparation and structure of vesicles: LUV detergent > LUV extrusion > MLV extrusion	Monroig et al. [18]
Krill PL (mainly PC, PE)	Maximal bioencapsulation is achieved: Nauplii densities: 300 nauplii ml ⁻¹ , number of doses of liposomes dispersion: single, product concentration: 0.5 g l ⁻¹	Monroig et al. [17]
1,2-PA-PC, Krill PL, soy PC, CHO	Types of liposomes, membrane composition (w/w) and findings: Encapsulation of vitamin A: Encapsulation of vitamin A: LUV: (98%)Krill PL(2%) vit. A LUV: (98%)Krill PL(2%) vit. A Increase of retinol content in <i>Artemia</i> nauplii Increase of retinol content in <i>Artemia</i> nauplii Encapsulation of methionine: LUV: 80% soy PC20% CHO or 80% 1,2-PA-PC 20% CHO MLV: 80% soy PC20%CHO Efficiency of methionine delivery to <i>Artemia</i> : MLV > LUV	Monroig et al. [19]
1,2-PA-PC, Krill PL, soy PC, CHO	Oxidative stability of formulated liposomes: (100%)Soy PC > (80%)1,2-PA-PC (20%)CHO > (80%)soy PC(20%)CHO > (100%)Krill PL (2%) vit A > (100%)Krill PL No size changes of liposomes during the experimental period	Monroig et al. [16]

1,2-PA-PC, dipalmitoyl phosphatidylcholine; PL, phospholipids; CHO, cholesterol; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; EFA, essential fatty acids; PG, phosphatidylglycerol; SS, Super Selco (Artemia Systems, INV E, Ghent) as control; PT, Tuna oil orbital oil emulsified with 12% herring roe polar lipids; L, liposomes with the composition, (40%)1,2-PA-PC (40%)PC(20%)CHO; PS, phosphatidylserine; CFE, cod fish extract; PHS, physiological saline; Toc, α -tocopherol

variety of enrichment products are available nowadays such as microalgae, microcapsules [158] and oil emulsion products [159].

PL especially MPL are considered to be a better way for providing EPA and DHA for larvae than TAG fish oil due to reasons such as: (1) marine fish larvae commonly ingest and assimilate better natural diets rich in PL than TAG [160–162]. The ratio of DHA:EPA in the PL naturally consumed by larvae is generally higher as compared to the corresponding ratio in TAG fish oil [156], (2) studies also showed that PL facilitate the absorption of lipids in the larvae gut [163] and thus promote growth and survival of larvae [164], and (3) PL have been shown to exert antioxidant properties against oxidation [87, 88].

Mcevoy et al. [14, 165] showed the advantage of using PC from soybean and marine fish eggs in enrichment of *Artemia* nauplii. They found that a mixture of DHA rich fish oil and PC (90:10) resulted in *Artemia* nauplii which were markedly enriched in DHA, and with minimal peroxidation in an aerated mixture during 18 h of enrichment. This is because the added PC functions as a natural emulsifying agent and a natural protectant against oxidation. They also showed that PC from marine egg sources was superior to soy PC in terms of n-3 PUFA content. This is presumably due to the presence of readily assimilable DHA and EPA in a ratio of 2:1 in marine roe lipids as compared to LA in soy PC. Their study corroborated the original work of Kanazawa et al. [166] using soy and bonito PC as feed supplements for larval sea bream and ayu.

As mentioned earlier, there are several forms of enrichment products commercially available nowadays for live prey. However, as compared to an emulsion, liposomes provide more advantages. This is due to their ability to encapsulate lipids as well as water soluble components. For example, liposomes have been successfully used to encapsulate vitamin C [19] or water soluble antibiotics [167] in *Artemia* nauplii enrichment. In addition, liposomes can also be used to encapsulate hydrophobic components such as vitamin A [19] and free amino acids such as methionine [19, 168] or glycine [169]. Many studies have also shown that it is possible to encapsulate considerable amounts of n-3 PUFA into liposomes for *Artemia* enrichment [14, 15, 170].

Future Prospects and Conclusion

MPL may offer more advantages to consumer, food, and aquaculture industries as compared to fish oils. Particularly, the use of MPL-based liposomes is expected to provide benefits such as better oxidative stability, higher bioavailability and higher fluidity as compared to other

PL-based liposomes. However, the use of MPL-based liposomes is just starting to be explored in both aquaculture and food industries and no current use of MPL-based liposomes for food applications has been reported. The next frontier in liposome application in the food industry will probably focus on the use of MPL for the development of n-3 PUFA enriched functional foods or the use of MPL-based liposomes as nutrient delivery system in foods and feed. Additionally, another area of study that needs further exploration is the use of liposomes for encapsulation of flavor, aroma and natural coloring compound in foods. However, due to the high content of n-3 PUFA in MPL, foods containing MPL are highly susceptible to lipid oxidation, which results in oxidative products that not only cause deterioration of food quality but also increase the risk of certain degenerative diseases as mentioned earlier. Therefore, it is expected that many more studies will be carried out in the future to explore the oxidative stability and sensory properties of MPL or MPL liposomes prior their potential uses in both food and aquaculture industries.

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References

- Okuyama H (2001) High n-6 to n-3 ratio of dietary fatty acids rather than serum cholesterol as a major risk factor for coronary heart disease. *Eur J Lipid Sci Technol* 103:418–422
- Hibbeln JR, Nieminen LRG, Blasbalg TL, Riggs JA, Lands WEM (2006) Healthy intakes of n-3 and n-6 fatty acids: estimations considering worldwide diversity. *Am J Clin Nutr* 83:1483S–1493S
- Simopoulos AP (2008) The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Exp Biol Med* 233:674–688
- Løvaas E (2006) Marine phospholipids (MPL) resources, applications and markets. In: Luten JB, Jacobsen C, Bekaert K, Saebo A, Oehlenschläger J (eds) *Seafood research from fish to dish*, 1st ed. edn. Wageningen Academic Publishers, The Netherlands, pp 17–28
- Neptune Technologies & Bioresources (2001) Natural phospholipids of marine origin containing flavonoids and polyunsaturated phospholipids and their uses [EP 1417211]
- Nara E, Miyashita K, Ota T (1997) Oxidative stability of liposomes prepared from soybean PC, chicken egg PC, and salmon egg PC. *Biosci Biotechnol Biochem* 61:1736–1738
- Nara E, Miyashita K, Ota T, Nadachi Y (1998) The oxidative stabilities of polyunsaturated fatty acids in salmon egg phosphatidylcholine liposomes. *Fish Sci* 64:282–286
- Araseki M, Yamamoto K, Miyashita K (2002) Oxidative stability of polyunsaturated fatty acid in phosphatidylcholine liposomes. *Biosci Biotechnol Biochem* 66:2573–2577
- Lyberg AM, Fasoli E, Adlercreutz P (2005) Monitoring the oxidation of docosahexaenoic acid in lipids. *Lipids* 40:969–979
- Jacobsen C (2008) Omega-3s in food emulsions: overview and case studies. *Agro Food Ind Hi Tech* 19:9–12

11. Rodriguez-Nogales JM, Perez-Mateos M, Busto MD (2004) Application of experimental design to the formulation of glucose oxidase encapsulation by liposomes. *J Chem Technol Biotechnol* 79:700–705
12. Xia SQ, Xu SY (2005) Ferrous sulfate liposomes: preparation, stability and application in fluid milk. *Food Res Int* 38:289–296
13. Taylor TM, Gaysinsky S, Davidson PM, Bruce BD, Weiss J (2007) Characterization of antimicrobial-bearing liposomes by zeta-potential, vesicle size, and encapsulation efficiency. *Food Biophys* 2:1–9
14. Mcevoy LA, Navarro JC, Hontoria F, Amat F, Sargent JR (1996) Two novel *Artemia* enrichment diets containing polar lipid. *Aquaculture* 144:339–352
15. Monroig O, Navarro JC, Amat I, Gonzalez P, Amat F, Hontoria F (2003) Enrichment of *Artemia* nauplii in PUFA, phospholipids, and water-soluble nutrients using liposomes. *Aquacult Int* 11:151–161
16. Monroig O, Navarro JC, Amat F, Gonzalez P, Hontoria F (2007) Oxidative stability and changes in the particle size of liposomes used in the *Artemia* enrichment. *Aquaculture* 266:200–210
17. Monroig O, Navarro JC, Amat F, Gonzalez P, Hontoria F (2006) Effects of nauplii density, product concentration and product dosage on the survival of the nauplii and EPA incorporation during *Artemia* enrichment with liposomes. *Aquaculture* 261:659–669
18. Monroig O, Navarro JC, Amat F, Gonzalez P, Bermejo A, Hontoria F (2006) Enrichment of *Artemia* nauplii in essential fatty acids with different types of liposomes and their use in the rearing of gilthead sea bream (*Sparus aurata*) larvae. *Aquaculture* 251:491–508
19. Monroig O, Navarro JC, Amat F, Hontoria F (2007) Enrichment of *Artemia* nauplii in vitamin A, vitamin C and methionine using liposomes. *Aquaculture* 269:504–513
20. Cansell M, Moussaoui N, Lefrançois C (2001) Stability of marine lipid-based-liposomes under acid conditions. Influence of xanthan gum. *J Liposome Res* 11:229–242
21. Cho SY, Joo DS, Choi HG, Nara E, Miyashita K (2001) Oxidative stability of lipids from squid tissues. *Fish Sci* 67:738–743
22. Mozuraityte R, Rustad T, Storro I (2006) Pro-oxidant activity of Fe²⁺ in oxidation of cod phospholipids in liposomes. *Eur J Lipid Sci Technol* 108:218–226
23. Mozuraityte R, Rustad T, Storro I (2006) Oxidation of cod phospholipids in liposomes: effects of salts, pH and zeta potential. *Eur J Lipid Sci Technol* 108:944–950
24. Mozuraityte R, Rustad T, Storro I (2008) The role of iron in peroxidation of polyunsaturated fatty acids in liposomes. *J Agric Food Chem* 56:537–543
25. Moriya H, Kuniminato T, Hosokawa M, Fukunaga K, Nishiyama T, Miyashita K (2007) Oxidative stability of salmon and herring roe lipids and their dietary effect on plasma cholesterol levels of rats. *Fish Sci* 73:668–674
26. Nacka F, Cansell M, Meleard P, Combe N (2001) Incorporation of alpha-tocopherol in marine lipid-based liposomes: in vitro and in vivo studies. *Lipids* 36:1313–1320
27. Nacka F, Cansell M, Gouygou JP, Gerbeaud C, Meleard P, Entressangles B (2001) Physical and chemical stability of marine lipid-based liposomes under acid conditions. *Colloids Surf B* 20:257–266
28. Nacka F, Cansell M, Entressangles B (2001) In vitro behavior of marine lipid-based liposomes, influence of pH, temperature, bile salts, and phospholipase A(2). *Lipids* 36:35–42
29. Arnaud JP (1995) Liposomes in the agro food-industry. *Agro Food Ind Hi Tech* 6:30–36
30. Falch E, Rustad T, Jonsdottir R, Shaw NB, Dumay J, Berge JP, Arason S, Kerry JP, Sandbakk M, Aursand M (2006) Geographical and seasonal differences in lipid composition and relative weight of by-products from gadiform species. *J Food Compost Anal* 19:727–736
31. Gbogouri GA, Linder M, Fanni J, Parmentier M (2006) Analysis of lipids extracted from salmon (*Salmo salar*) heads by commercial proteolytic enzymes. *Eur J Lipid Sci Technol* 108:766–775
32. Striby L, Lafont R, Goux M (1999) Improvement in the Iatroscan thin-layer chromatographic-flame ionisation detection analysis of marine lipids. Separation and quantitation of monoacylglycerols and diacylglycerols in standards and natural samples. *J Chromatogr A* 849:371–380
33. Medina I, Aubourg SP, Martin RP (1995) Composition of phospholipids of white muscle of 6 tuna species. *Lipids* 30:1127–1135
34. Body DR, Vlieg P (1989) Distribution of the lipid classes and eicosapentaenoic (20-5) and docosahexaenoic (22-6) acids in different sites in blue mackerel (*Scomber australasicus*) filets. *J Food Sci* 54:569–572
35. Hazel JR (1985) Determination of the phospholipid-composition of trout gill by Iatroscan TLC/FID—effect of thermal-acclimation. *Lipids* 20:516–520
36. Schneider M (2008) Major sources, composition and processing. In: Gunstone FD (ed) *Phospholipid technology and applications*. The Oily Press, Bridgewater, pp 21–40
37. Saito H, Kotani Y, Keriko JM, Xue CH, Taki K, Ishihara K, Ueda T, Miyata S (2002) High levels of n-3 polyunsaturated fatty acids in *Euphausia pacifica* and its role as a source of docosahexaenoic and icosapentaenoic acids for higher trophic levels. *Mar Chem* 78:9–28
38. Le Grandois J, Marchioni E, Zhao MJ, Giuffrida F, Ennahar S, Bindler F (2009) Investigation of natural phosphatidylcholine sources: separation and identification by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS2) of molecular species. *J Agric Food Chem* 57:6014–6020
39. Boselli E, Caboni MF (2000) Supercritical carbon dioxide extraction of phospholipids from dried egg yolk without organic modifier. *J Supercrit Fluids* 19:45–50
40. Kang KY, Ahn DH, Jung SM, Kim DH, Chun BS (2005) Separation of protein and fatty acids from tuna viscera using supercritical carbon dioxide. *Biotechnol Bioprocess Eng* 10:315–321
41. Letisse M, Rozieres M, Hiol A, Sergent M, Comeau L (2006) Enrichment of EPA and DHA from sardine by supercritical fluid extraction without organic modifier—I. Optimization of extraction conditions. *J Supercrit Fluids* 38:27–36
42. Aro H, Jarvenpaa E, Konko K, Sihvonen M, Hietaniemi V, Huopalahti R (2009) Isolation and purification of egg yolk phospholipids using liquid extraction and pilot-scale supercritical fluid techniques. *Eur J Lipid Sci Technol* 228:857–863
43. Froning GW, Wehling RL, Cuppett SL, Pierce MM, Niemann L, Siekman DK (1990) Extraction of cholesterol and other lipids from dried egg-yolk using supercritical carbon dioxide. *J Food Sci* 55:95–98
44. Rossi M, Spedicato E, Shiraldi A (1990) Improvement of supercritical carbon dioxide extraction of egg lipids by means of ethanolic entrainer. *Ital J Food Sci* 2:249–256
45. Lemaître-Delaunay D, Pachiaudi C, Laville M, Pousin J, Armstrong M, Lagarde M (1999) Blood compartmental metabolism of docosahexaenoic acid (DHA) in humans after ingestion of a single dose of [C⁻¹³]DHA in phosphatidylcholine. *J Lipid Res* 40:1867–1874
46. Amate L, Gil A, Ramirez M (2001) Feeding infant piglets formula with long-chain polyunsaturated fatty acids as triacylglycerols or phospholipids influences the distribution of these fatty acids in plasma lipoprotein fractions. *J Nutr* 131:1250–1255

47. Wijendran V, Huang MC, Diau GY, Boehm G, Nathanielsz PW, Brenna JT (2002) Efficacy of dietary arachidonic acid provided as triglyceride or phospholipid as substrates for brain arachidonic acid accretion in baboon neonates. *Pediatr Res* 51:265–272
48. Peng JL, Larondelle Y, Pham D, Ackman RG, Rollin X (2003) Polyunsaturated fatty acid profiles of whole body phospholipids and triacylglycerols in anadromous and landlocked Atlantic salmon (*Salmo salar* L.) fry. *Comp Biochem Phys B* 134:335–348
49. Phares Pharmaceutical Research N.V. (2004) Marine Lipid Compositions [WO/2004/047554]
50. Narayan B, Miyashita K, Hosakawa M (2006) Physiological effects of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)—a review. *Food Rev Int* 22:291–307
51. Leaf A (2008) Historical overview of n-3 fatty acids and coronary heart disease. *Am J Clin Nutr* 87:1978S–1980S
52. Virtanen JK, Mozaffarian D, Chiuve SE, Rimm EB (2008) Fish consumption and risk of major chronic disease in men. *Am J Clin Nutr* 88:1618–1625
53. Calon F, Cicchetti F (2009) Omega-3 fatty acid in Parkinson disease. *Agro Food Ind Hi Tech* 20:7–9
54. Fotuhi M, Mohassel P, Yaffe K (2009) Fish consumption, long-chain omega-3 fatty acids and risk of cognitive decline or Alzheimer disease: a complex association. *Nat Clin Pract Neurol* 5:140–152
55. Ramakrishnan U, Imhoff-Kunsch B, DiGirolamo AM (2009) Role of docosahexaenoic acid in maternal and child mental health. *Am J Clin Nutr* 89:958S–962S
56. Boudraut C, Bazinet RP, Ma DWL (2009) Experimental models and mechanisms underlying the protective effects of n-3 polyunsaturated fatty acids in Alzheimer's disease. *J Nutr Biochem* 20:1–10
57. Tinoco SMB, Sichieri R, Setta CL, Moura AS, Do Carmo MGT (2009) n-3 polyunsaturated fatty acids in milk is associate to weight gain and growth in premature infants. *Lipids Health Dis* 8:23
58. Adkins Y, Kelley DS (2010) Mechanisms underlying the cardioprotective effects of omega-3 polyunsaturated fatty acids. *J Nutr Biochem* 21:781–792
59. Lopez-Huertas E (2010) Health effects of oleic acid and long chain omega-3 fatty acids (EPA and DHA) enriched milks. A review of intervention studies. *Pharmacol Res* 61:200–207
60. Wilson TA, Meservy CM, Nicolosi RJ (1998) Soy lecithin reduces plasma lipoprotein cholesterol and early atherogenesis in hypercholesterolemic monkeys and hamsters: beyond linoleate. *Atherosclerosis* 140:147–153
61. Zeisel SH (1992) Choline—an important nutrient in brain-development, liver-function and carcinogenesis. *J Am Coll Nutr* 11:473–481
62. Pharmacia AB (1995) Phospholipids containing omega-3 fatty acids [US Patent 5434183]
63. Bunea R, El Farrah K, Deutsch L (2004) Evaluation of the effects of Neptune krill oil on the clinical course of hyperlipidemia. *Altern Med Rev* 9:420–428
64. Maki KC, Reeves MS, Farmer M, Griinari M, Berge K, Vik H, Hubacher R, Rains TM (2009) Krill oil supplementation increases plasma concentrations of eicosapentaenoic and docosahexaenoic acids in overweight and obese men and women. *Nutr Res* 29:609–615
65. Tandy S, Chung RWS, Wat E, Kamili A, Berge K, Griinari M, Cohn JS (2009) Dietary krill oil supplementation reduces hepatic steatosis, glycaemia, and hypercholesterolemia in high-fat-fed mice. *J Agric Food Chem* 57:9339–9345
66. Deutsch L (2007) Evaluation of the effect of Neptune krill oil on chronic inflammation and arthritic symptoms. *J Am Coll Nutr* 26:39–48
67. Sampalis F, Bunea R, Pelland MF, Kowalski O, Duguet N, Dupuis S (2003) Evaluation of the effects of Neptune krill oil on the management of premenstrual syndrome and dysmenorrhea. *Altern Med Rev* 8:171–179
68. Ierna M, Kerr A, Scales H, Berge K, Griinari M (2010) Supplementation of diet with krill oil protects against experimental rheumatoid arthritis. *BMC Musculoskelet Disorders* 11:136
69. Hayashi H, Tanaka Y, Hibino H, Umeda Y, Kawamitsu H, Fujimoto H, Amakawa T (1999) Beneficial effect of salmon roe phosphatidylcholine in chronic liver disease. *Curr Med Res Opin* 15:177–184
70. Taylor LA, Pletschen L, Arends J, Unger C, Massing U (2010) Marine phospholipids—a promising new dietary approach to tumor-associated weight loss. *Support Care Cancer* 18:159–170
71. Lasch J, Weissing V, Brandi M (2003) Preparation of liposomes. In: Torchilin VP, Weissing V (eds) *Liposomes: a practical approach*, 2nd edn. Oxford University Press, New York, pp 3–30
72. Lasic DD (1998) Novel applications of liposomes. *Trends Biotechnol* 16:307–321
73. Watwe RM, Bellare JR (1995) Manufacture of liposomes—a review. *Curr Sci* 68:715–724
74. Kim HY, Baia IC (1991) Novel liposome microencapsulation techniques for food applications. *Trends Food Sci Tech* 2:55–61
75. Fruhwirth GO, Loidl A, Hermetter A (2007) Oxidized phospholipids: from molecular properties to disease. *BBA Mol Basis Dis* 1772:718–736
76. Domingues MRM, Reis A, Domingues P (2008) Mass spectrometry analysis of oxidized phospholipids. *Chem Phys Lipids* 156:1–12
77. Subbanagounder G, Deng YJ, Borromeo C, Dooley AN, Beliner JA, Salomon RG (2002) Hydroxy alkenal phospholipids regulate inflammatory functions of endothelial cells. *Vascul Pharmacol* 38:201–209
78. Leitinger N (2003) Oxidized phospholipids as modulators of inflammation in atherosclerosis. *Curr Opin Lipidol* 14:421–430
79. Leitinger N (2005) Oxidized phospholipids as triggers of inflammation in atherosclerosis. *Mol Nutr Food Res* 49:1063–1071
80. Spickett CM, Dever G (2005) Studies of phospholipid oxidation by electrospray mass spectrometry: from analysis in cells to biological effects. *Biofactors* 24:17–31
81. Spiteller G (2006) Peroxyl radicals: inducers of neurodegenerative and other inflammatory diseases. Their origin and how they transform cholesterol, phospholipids, plasmalogens, polyunsaturated fatty acids, sugars, and proteins into deleterious products. *Free Radic Bio Med* 41:362–387
82. Bochkov VN (2007) Inflammatory profile of oxidized phospholipids. *Thromb Haemost* 97:348–354
83. Imal Y, Kuba K, Neely GG, Yaghubian-Malhami R, Perkmann T, van Loo G, Ermolaeva M, Veldhuizen R, Leung YHC, Wang H, Liu H, Sun Y, Pasparakis M, Kopf M, Mech C, Bavari S, Peiris JS, Slutsky AS, Akira S, Hultqvist M, Hölm Dahl R, Nicholls J, Jiang C, Binder CJ, Penninger JM (2008) Identification of oxidative stress and toll-like receptor 4 signaling as a key pathway of acute lung injury. *Cell* 133:235–249
84. Subbanagounder G, Leitinger N, Shih PT, Faull KF, Berliner JA (1999) Evidence that phospholipid oxidation products and/or platelet-activating factor play an important role in early atherogenesis—in vitro and in vivo inhibition by WEB 2086. *Circ Res* 85:311–318
85. Androulakis N, Durand H, Ninio E, Tsoukatos DC (2005) Molecular and mechanistic characterization of platelet-activating factor-like bioactivity produced upon LDL oxidation. *J Lipid Res* 46:1923–1932
86. Gopfert MS, Siedler F, Siess W, Sellmayer A (2005) Structural identification of oxidized acyl-phosphatidylcholines that induce platelet activation. *J Vasc Res* 42:120–132

87. King MF, Boyd LC, Sheldon BW (1992) Effects of phospholipids on lipid oxidation of a salmon oil model system. *J Am Oil Chem Soc* 69:237–242
88. King MF, Boyd LC, Sheldon BW (1992) Antioxidant properties of individual phospholipids in a salmon oil model system. *J Am Oil Chem Soc* 69:545–551
89. Boyd LC, Nwosu VC, Young CL, MacMillan L (1998) Monitoring lipid oxidation and antioxidant effects of phospholipids by headspace gas chromatographic analyses of rancimat trapped volatiles. *J Food Lipids* 5:269–282
90. Miyashita K, Nara E, Ota T (1994) Comparative study on the oxidative stability of phosphatidylcholines from salmon egg and soybean in an aqueous solution. *Biosci Biotechnol Biochem* 58:1772–1775
91. Applegate KR, Glomset JA (1986) Computer-based modeling of the conformation and packing properties of docosahexaenoic acid. *J Lipid Res* 27:658–680
92. Applegate KR, Glomset JA (1991) Effect of acyl chain unsaturation on the conformation of model diacylglycerols—a computer modeling study. *J Lipid Res* 32:1635–1644
93. Applegate KR, Glomset JA (1991) Effect of acyl chain unsaturation on the packing of model diacylglycerols in simulated monolayers. *J Lipid Res* 32:1645–1655
94. Feng SS, Brockman HL, Macdonald RC (1994) On osmotic-type equations of state for liquid-expanded monolayers of lipids at the air–water interface. *Langmuir* 10:3188–3194
95. Brockman HL, Applegate KR, Momsen MM, King WC, Glomset JA (2003) Packing and electrostatic behavior of *sn*-2-docosahexaenoyl and -arachidonoyl phosphoglycerides. *Biophys J* 85:2384–2396
96. Albrand M, Pageaux JF, Lagarde M, Dolmazon R (1994) Conformational analysis of isolated docosahexaenoic acid (22/6 N-3) and its 14-(S) and 11-(S) hydroxy derivatives by force-field calculations. *Chem Phys Lipids* 72:7–17
97. Koenig BW, Strey HH, Gawrisch K (1997) Membrane lateral compressibility determined by NMR and X-ray diffraction: effect of acyl chain polyunsaturation. *Biophys J* 73:1954–1966
98. Eldho NV, Feller SE, Tristram-Nagle S, Polozov IV, Gawrisch K (2003) Polyunsaturated docosahexaenoic vs docosapentaenoic acid—differences in lipid matrix properties from the loss of one double bond. *J Am Chem Soc* 125:6409–6421
99. Feller SE, Gawrisch K, MacKerell AD (2002) Polyunsaturated fatty acids in lipid bilayers: intrinsic and environmental contributions to their unique physical properties. *J Am Chem Soc* 124:318–326
100. Saiz L, Klein ML (2001) Structural properties of a highly polyunsaturated lipid bilayer from molecular dynamics simulations. *Biophys J* 81:204–216
101. Huber T, Rajamoorthi K, Kurze VF, Beyer K, Brown MF (2002) Structure of docosahexaenoic acid-containing phospholipid bilayers as studied by H-2 NMR and molecular dynamics simulations. *J Am Chem Soc* 124:298–309
102. Everts S, Davis JH (2000) H⁻¹ and C⁻¹³ NMR of multilamellar dispersions of polyunsaturated (22:6) phospholipids. *Biophys J* 79:885–897
103. Gawrisch K, Eldho NV, Holte LL (2003) The structure of DHA in phospholipid membranes. *Lipids* 38:445–452
104. Bandarra NM, Campos RM, Batista I, Nunes ML, Empis JM (1999) Antioxidant synergy of alpha-tocopherol and phospholipids. *J Am Oil Chem Soc* 76:905–913
105. Ohshima T, Fujita Y, Koizumi C (1993) Oxidative stability of sardine and mackerel lipids with reference to synergism between phospholipids and alpha-tocopherol. *J Am Oil Chem Soc* 70:269–276
106. Saito H, Ishihara K (1997) Antioxidant activity and active sites of phospholipids as antioxidants. *J Am Oil Chem Soc* 74:1531–1536
107. Kashima M, Cha GS, Isoda Y, Hirano J, Miyazawa T (1991) The antioxidant effects of phospholipids on perilla oil. *J Am Oil Chem Soc* 68:119–122
108. Hildebrand DH, Terao J, Kito M (1984) Phospholipids plus tocopherols increase soybean oil stability. *J Am Oil Chem Soc* 61:552–555
109. Weng XC, Gordon MH (1993) Antioxidant synergy between phosphatidyl ethanolamine and alpha-tocopherylquinone. *Food Chem* 48:165–168
110. Mei LY, Decker EA, McClements DJ (1998) Evidence of iron association with emulsion droplets and its impact on lipid oxidation. *J Agric Food Chem* 46:5072–5077
111. Kuzuya M, Yamada K, Hayashi T, Funaki C, Naito M, Asai K, Kuzuya F (1991) Oxidation of low-density-lipoprotein by copper and iron in phosphate buffer. *Biochim Biophys Acta* 1084:198–201
112. Djuric Z, Potter DW, Taffe BG, Strasburg GM (2001) Comparison of iron-catalyzed DNA and lipid oxidation. *J Biochem Mol Toxicol* 15:114–119
113. Were LM, Bruce BD, Davidson PM, Weiss J (2003) Size, stability, and entrapment efficiency of phospholipid nanocapsules containing polypeptide antimicrobials. *J Agric Food Chem* 51:8073–8079
114. Laridi R, Kheadr EE, Benech RO, Vuilleumard JC, Lacroix C, Fliss I (2003) Liposome encapsulated nisin Z: optimization, stability and release during milk fermentation. *Int Dairy J* 13:325–336
115. Sulkowski WW, Pentak D, Nowak K, Sulkowska A (2005) The influence of temperature, cholesterol content and pH on liposome stability. *J Mol Struct* 744:737–747
116. Finean JB (1990) Interaction between cholesterol and phospholipid in hydrated bilayers. *Chem Phys Lipids* 54:147–156
117. Fiorentini D, Landi L, Barzanti V, Cabrini L (1989) Buffers can modulate the effect of sonication on egg lecithin liposomes. *Free Radic Res Commun* 6:243–250
118. Kirby C, Clarke J, Gregoriadis G (1980) Effect of the cholesterol content of small unilamellar liposomes on their stability in vivo and in vitro. *Biochem J* 186:591–598
119. Senior J, Gregoriadis G (1982) Stability of small unilamellar liposomes in serum and clearance from the circulation—the effect of the phospholipid and cholesterol components. *Life Sci* 30:2123–2136
120. Papahadj D, Jacobson K, Nir S, Isac T (1973) Phase-transitions in phospholipid vesicles—fluorescence polarization and permeability measurements concerning effect of temperature and cholesterol. *Biochim Biophys Acta* 311:330–348
121. Brockerh H (1974) Model of interaction of polar lipids, cholesterol, and proteins in biological-membranes. *Lipids* 9:645–650
122. Huang CH (1977) Structural model for cholesterol–phosphatidylcholine complexes in bilayer membranes. *Lipids* 12:348–356
123. Frankel EN (1993) In search of better methods to evaluate natural antioxidants and oxidative stability in food lipids. *Trends Food Sci Tech* 4:220–225
124. Cillard J, Cillard P, Cormier M, Girre L (1980) Alpha-tocopherol prooxidant effect in aqueous-media—increased autooxidation rate of linoleic-acid. *J Am Oil Chem Soc* 57:252–255
125. Bazin BC, Cillard J, Koskas JP, Cillard P (1984) Arachidonic acid autooxidation in an aqueous media effect of α -tocopherol, cystein and nucleic acids. *J Am Oil Chem Soc* 61:1212–1215
126. MacMillan DK, Murphy RC (1995) Analysis of lipid hydroperoxides and long-chain conjugated keto acids by negative ion

- electrospray mass spectrometry. *J Am Soc Mass Spectrom* 6:1190–1201
127. Spickett CM, Pitt AR, Brown AJ (1998) Direct observation of lipid hydroperoxides in phospholipid vesicles by electrospray mass spectrometry. *Free Radical Biol Med* 25:613–620
 128. Spickett CM, Rennie N, Winter H, Zambonin L, Landi L, Jerlich A, Schaur RJ, Pitt AR (2001) Detection of phospholipid oxidation in oxidatively stressed cells by reversed-phase HPLC coupled with positive-ionization electroscopy MS. *Biochem J* 355:449–457
 129. Yin HY, Cox BE, Liu W, Porter NA, Morrow JD, Milne GL (2009) Identification of intact oxidation products of glycerophospholipids in vitro and in vivo using negative ion electrospray ion trap mass spectrometry. *J Mass Spectrom* 44:672–680
 130. Taylor TM, Davidson PM, Bruce BD, Weiss J (2005) Liposomal nanocapsules in food science and agriculture. *Crit Rev Food Sci* 45:587–605
 131. Thompson AK, Hindmarsh JP, Haisman D, Rades T, Singh H (2006) Comparison of the structure and properties of liposomes prepared from milk fat globule membrane and soy phospholipids. *J Agric Food Chem* 54:3704–3711
 132. Thompson AK, Haisman D, Singh H (2006) Physical stability of liposomes prepared from milk fat globule membrane and soya phospholipids. *J Agric Food Chem* 54:6390–6397
 133. Thompson AK, Mozafari MR, Singh H (2007) The properties of liposomes produced from milk fat globule membrane material using different techniques. *Lait* 87:349–360
 134. Cansell M, Nacka F, Combe N (2003) Marine lipid-based liposomes increase in vivo FA bioavailability. *Lipids* 38:551–559
 135. Cansell M, Moussaoui N, Petit AP, Denizot A, Combe N (2006) Feeding rats with liposomes or fish oil differently affects their lipid metabolism. *Eur J Lipid Sci Technol* 108:459–467
 136. Hossain Z, Kurihara H, Hosokawa M, Takahashi K (2006) Docosahexaenoic acid and eicosapentaenoic acid-enriched phosphatidylcholine liposomes enhance the permeability, transportation and uptake of phospholipids in Caco-2 cells. *Mol Cell Biochem* 285:155–163
 137. Kirby CJ, Whittle CJ, Rigby N, Coxon DT, Law BA (1991) Stabilization of ascorbic-acid by microencapsulation in liposomes. *Int J Food Sci Tech* 26:437–449
 138. Chang HM, Lee YC, Chen CC, Tu YY (2002) Microencapsulation protects immunoglobulin in yolk (IgY) specific against *Helicobacter pylori* urease. *J Food Sci* 67:15–20
 139. Rao DR, Chawan CB, Veeramachaneni R (1995) Liposomal encapsulation of beta-galactosidase—comparison of 2 methods of encapsulation and in vitro lactose digestibility. *J Food Biochem* 18:239–251
 140. Hsieh YF, Chen TL, Wang YT, Chang JH, Chang HM (2002) Properties of liposomes prepared with various lipids. *J Food Sci* 67:2808–2813
 141. Lee SC, Yuk HG, Lee DH, Lee KE, Hwang YI, Ludescher RD (2002) Stabilization of retinol through incorporation into liposomes. *J Biochem Mol Biol* 35:358–363
 142. Lee SK, Han JH, Decker EA (2002) Antioxidant activity of phosphatidylcholine liposomes and meat model systems. *J Food Sci* 67:37–41
 143. Lee SC, Lee KE, Kim JJ, Lim SH (2005) The effect of cholesterol in the liposome bilayer on the stabilization of incorporated retinol. *J Liposome Res* 15:157–166
 144. Thapon JL, Brule G (1986) Effects of pH and ionic-strength on lysozyme-caseins affinity. *Lait* 66:19–30
 145. Arnaud JP (1998) Liposome-based functional drinks. *Agro Food Ind Hi Tech* 9:37–40
 146. Alkhalaf W, Piard JC, Elsoda M, Gripon JC, Desmazeaud M, Vassal L (1988) Liposomes as proteinase carriers for the accelerated ripening of Saint-Paulin type cheese. *J Food Sci* 53:1674–1679
 147. Kirby CJ, Brooker BE, Law BA (1987) Accelerated ripening of cheese using liposome-encapsulated enzyme. *Int J Food Sci Tech* 22:355–375
 148. Picon A, Gaya P, Medina M, Nunez M (1994) The effect of liposome encapsulation of chymosin derived by fermentation on Manchego cheese ripening. *J Dairy Sci* 77:16–23
 149. Picon A, Gaya P, Medina M, Nunez M (1997) Proteinases encapsulated in stimulated release liposomes for cheese ripening. *Biotechnol Lett* 19:345–348
 150. Benech RO, Kheadr EE, Laridi R, Lacroix C, Fliss I (2002) Inhibition of *Listeria innocua* in Cheddar cheese by addition of nisin Z in liposomes or by in situ production in mixed culture. *Appl Environ Microbiol* 68:3683–3690
 151. Kheadr EE, Vuillelard JC, El Deeb SA (2000) Accelerated Cheddar cheese ripening with encapsulated proteinases. *Int J Food Sci Tech* 35:483–495
 152. Matsuzaki M, McCafferty F, Karel M (1989) The effect of cholesterol content of phospholipid-vesicles on the encapsulation and acid resistance of beta-galactosidase from *Escherichia coli*. *Int J Food Sci Technol* 24:451–460
 153. Banville C, Vuillelard JC, Lacroix C (2000) Comparison of different methods for fortifying Cheddar cheese with vitamin D. *Int Dairy J* 10:375–382
 154. Navarro JC, Bell MV, Amat F, Sargent JR (1992) The fatty-acid composition of phospholipids from brine shrimp, *Artemia* sp., eyes. *Comp Biochem Physiol B* 103:89–91
 155. Bell MV, Batty RS, Dick JR, Fretwell K, Navarro JC, Sargent JR (1995) Dietary deficiency of docosahexaenoic acid impairs vision at low-light intensities in Juvenile herring (*Clupea harengus* L.). *Lipids* 30:443–449
 156. Sargent JR, Mcevoy LA, Bell JG (1997) Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. *Aquaculture* 155:117–127
 157. Navarro JC, Amat F, Sargent JR (1992) Lipid-composition of cysts of the brine shrimp *Artemia* sp. from Spanish populations. *J Exp Mar Biol Ecol* 155:123–131
 158. Southgate PC, Lou DC (1995) Improving the eta-3 Hufa composition of *Artemia* using microcapsules containing marine oils. *Aquaculture* 134:91–99
 159. Narciso L, Pousao-Ferreira P, Passos A, Luis O (1999) HUFA content and DHA/EPA improvements of *Artemia* sp. with commercial oils during different enrichment periods. *Aquac Res* 30:21–24
 160. Salhi M, Hernandez-Cruz CM, Bessonart M, Izquierdo MS, Fernandez-Palacios H (1999) Effect of different dietary polar lipid levels and different n-3 HUFA content in polar lipids on gut and liver histological structure of gilthead seabream (*Sparus aurata*) larvae. *Aquaculture* 179:253–263
 161. Izquierdo MS, Tandler A, Salhi M, Kolkovski S (2001) Influence of dietary polar lipids' quantity and quality on ingestion and assimilation of labelled fatty acids by larval gilthead seabream. *Aquacult Nutr* 7:153–160
 162. Cahu CL, Infante JLZ, Barbosa V (2003) Effect of dietary phospholipid level and phospholipid: neutral lipid value on the development of sea bass (*Dicentrarchus labrax*) larvae fed a compound diet. *Br J Nutr* 90:21–28
 163. Koven W, Barr Y, Hadas E, Ben-Atia I, Chen Y, Weiss R, Tandler A (1999) The potential of liposomes as a nutrient supplement in first-feeding marine fish larvae. *Aquacult Nutr* 5:251–256
 164. Kanazawa A, Teshima SI, Sakamoto M (1985) Effects of dietary lipids, fatty-acids, and phospholipids on growth and survival of prawn (*Penaeus japonicus*) larvae. *Aquaculture* 50:39–49

165. Mcevoy LA, Navarro JC, Amat F, Sargent JR (1997) Application of soya phosphatidylcholine in tuna orbital oil enrichment emulsions for *Artemia*. *Aquacult Int* 5:517–526
166. Kanazawa A, Teshima S, Sakamoto M (1985) Effects of dietary bonito-egg phospholipids and some phospholipids on growth and survival of the larval ayu, *Plecoglossus altivelis*. *Z Angew Ichthyol* 4:156–170
167. Touraki M, Rigas P, Kastritsis C (1995) Liposome mediated delivery of water soluble antibiotics to the larvae of aquatic animals. *Aquaculture* 136:1–10
168. Tonheim SK, Koven W, Ronnestad I (2000) Enrichment of *Artemia* with free methionine. *Aquaculture* 190:223–235
169. Ozkizilcik S, Chu FLE (1994) Uptake and metabolism of liposomes by *Artemia* nauplii. *Aquaculture* 128:131–141
170. Hontoria F, Crowe JH, Crowe LM, Amat F (1994) Potential use of liposomes in larviculture as a delivery system through *Artemia* nauplii. *Aquaculture* 127:255–264

PAPER II

Lu, F. S. H., Nielsen, N. S., Baron, C. P., Jensen, L. H. S., & Jacobsen, C.

Physico-chemical properties of marine phospholipid emulsions.

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Physico-chemical Properties of Marine Phospholipid Emulsions

F. S. H. Lu · N. S. Nielsen · C. P. Baron ·
L. H. S. Jensen · C. Jacobsen

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Abstract Many studies have shown that marine phospholipids (PL) have better bioavailability, better resistance towards oxidation and contain higher polyunsaturated fatty acids such as eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) than triglycerides (TAG) present in fish oil. The objective of this study was to investigate the emulsifying properties of various commercial marine PL and the feasibility of using them to prepare stable emulsions prepared with or without addition of fish oil. In addition, this study also investigated the relationship between chemical composition of marine PL and the stability of their emulsions. Physical stability was investigated through particle size distribution (PSD), zeta potential, microscopy inspection and emulsion separation (ES); while the oxidative and hydrolytic stability of emulsions were investigated through peroxide value (PV) and free fatty acids value (FFA) after 32 days storage at room temperature and at 2 °C. In conclusion, marine PL showed good emulsifying properties and it was possible to prepare marine PL emulsions with and without addition of fish oil. Emulsion with both good oxidative stability and physical stability could be prepared by using marine PL of high purity, less TAG, more PL, cholesterol and higher antioxidant content.

Keywords Physicochemical properties · Marine phospholipids · Fish oil · Emulsion stability · Oxidative stability · Particle size distribution

Introduction

Marine phospholipids (PL) have received much attention recently, especially on issues related to their health benefits and antioxidative properties. As far as the health benefits are concerned, many studies have shown that marine PL provide more advantages than triglycerides (TAG) present in fish oil. These advantages include a higher content of health beneficial n-3 polyunsaturated fatty acids (PUFA), particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [1] and better bioavailability [2]. Several studies have also shown that marine PL have antioxidative properties [3, 4]. Health benefits and oxidative stability of marine PL have been reviewed extensively in a previous publication [5] and will therefore not be discussed further in this paper.

Several studies on food fortification with n-3 PUFA from fish oil have been reported by Jacobsen [6], but no information about food fortification with marine PL is available in the literature. Nevertheless, increasing knowledge regarding the health benefits of marine PL has led to growing awareness about the potential of using marine PL as ingredient for food fortification. PL in general have good emulsifying properties and are potential natural surfactants that can be used to prepare emulsions. This is due to their unique molecular structure that contains both lipophilic fatty acid groups and a hydrophilic head group. Emulsions can be used as effective carriers of n-3 PUFA rich oil because they can easily be incorporated into aqueous and emulsified foods. Moreover, by manipulation

F. S. H. Lu · N. S. Nielsen · C. P. Baron ·
L. H. S. Jensen · C. Jacobsen (✉)
Division of Industrial Food Research, Lipid and Oxidation
Group, National Food Institute, Technical University
of Denmark, Søtofts Plads, Building 221,
2800 Kgs. Lyngby, Denmark
e-mail: chja@food.dtu.dk

F. S. H. Lu
e-mail: fshl@food.dtu.dk

of the physico-chemical characteristics of the emulsion, its oxidative stability can be increased [7]. These physical characteristics include the particle size distribution, fat content, type and ratio of emulsifier to fat, physical state of the emulsion droplets, the characteristics of the interfacial membrane, etc..

Generally, emulsions are thermodynamically unstable systems and they tend to break down over time. These breakdown processes include creaming, sedimentation, flocculation, coalescence, Ostwald-ripening and phase inversion [7, 8]. Asai and Watanabe [9] have investigated the dispersal mechanism of sesame oil in soybean phosphatidyl choline (PC) to form o/w emulsion by using sonication, PC was chosen as it was known for its superior emulsifying properties [10]. The dispersal mechanism was evaluated by characterizing the dispersed particles through dynamic light scattering, fluorescence spectroscopy and surface monolayer techniques (measurement of collapse and spreading pressures). Their study showed that a stable dispersion was not obtained when the PC mole fraction was <0.03 (or oil fraction >0.95). This is because the PC monolayer did not cover the oil droplets completely and this led to a drastic increase in droplet sizes and consequently separation into oil and water occurred. They recommended oil fractions of 0–0.8 in order to obtain a stable PC o/w dispersion. In addition, they reported that the coexistence of PL-monolayer encased oil droplets and a PL bilayer (liposomes) are crucial to stabilizing this kind of o/w emulsion as the PL bilayer has a maximum value of spreading pressure [9].

We therefore hypothesized that the physical stability of marine PL emulsions varies depending on the ratio of oil and PL, and the type of PL as surfactant, i.e. the chemical composition of marine PL used for emulsion preparation. Thus, the main goal of this study was to investigate the emulsifying properties of marine PL and to formulate physically stable emulsions with appropriate amount of marine PL and fish oil. Apart from emulsion stability, the physico-chemical properties and microstructure of the resulting marine PL emulsions were also determined. In order to get an indication of the oxidative and hydrolytic stability of marine PL emulsions, peroxide value (PV) and free fatty acids (FFA) were determined on the samples before and after storage. In the final part of this study, we studied the relationship between the chemical composition of the raw materials and the stability of their emulsions.

Materials

Three different marine phospholipid preparations (LC, MPT and MPL) were obtained from PhosphoTech Laboratoires (Saint-Herblain Cedex, France), University of

Tromsø (Tromsø, Norway) and Triple Nine (Esbjerg, Denmark), respectively. Fish oil (Maritex 43-01) was supplied by Maritex A/S, subsidiary of TINE, BA (Sortland, Norway). This fish oil had low initial PV (0.16 mequiv/kg) and comprised 240.4 mg/kg α -tocopherol, 99.3 mg/kg γ -tocopherol and 37.9 mg/kg δ -tocopherol. The chemicals, sodium acetate and imidazole were obtained from Fluka (Sigma-Aldrich Chemie GmbH, Buchs, Spain) and Merck (Darmstadt, Germany), respectively. Other solvents were of HPLC grade (Lab-Scan, Dublin, Ireland).

Methods

Determination of Chemical Composition of Marine PL

Determination of Lipid Classes by Thin Layer Chromatography

The different lipid classes of marine PL were measured by TLC–FID Iatroscan MK-V (Iatron Laboratories, Inc., Tokyo, Japan) with Chromo Star v3.24S software (Bruker-Franzen & SCAP, Germany). The ten silica gel chromarods SIII (Iatron Laboratories Inc., Tokyo, Japan) were blank scanned twice immediately before sample application in order to remove any impurities. Lipids (10–20 mg/mL chloroform methanol, 2:1) were then spotted on the chromarods using semi-automatic sample spotter (SES GmbH—Analyse Systeme, Germany). The quantification of lipid classes was done by development in n-heptane/diethyl ether/formic acid (70:10:0.02, vol/vol/vol). The neutral lipids (NL) consisting of triglycerides (TAG), free fatty acids (FFA) and cholesterol (CHO) were separated from polar lipids and non-lipid material. After development, the rods were dried in an oven at 120 °C for 2 min and then fully scanned in Iatroscan MK-V. The air and hydrogen flow rates were set at 200 L/min and 160 mL/min, respectively. The scan speed was set at 30 s/rod. Lipid composition of marine PL was expressed as mean percentage of three analyses from each sample.

Determination of Fatty Acid and Phospholipids Composition

For fatty acids composition, approximately 0.5 mL marine phospholipids in chloroform (with a concentration of 10–20 mg/mL) was transferred to a Sep-pak column containing 500 mg aminopropyl-modified silica (Waters Corporation, Milford, MA, USA) for lipid separation. A mixture of 2 × 2 mL chloroform and 2-propanol (ratio 2:1) was used to elute the neutral lipid fraction (NL) whereas 3 × 2 mL methanol was used to elute PL fraction

by gravity. Eluates were evaporated under nitrogen and methylated according to AOCS Official Method Ce 2-66 [11], followed by separation through gas chromatography (HP 5890 A, Hewlett Packard, Palo Alto, CA) with a OMEGAWAX™ 320 column according to the method described by AOCS Official Method Ce 1b-89 [12]. The analyses were performed in duplicate. PL composition of marine PL was determined through ³¹P NMR by Spectra Service GmbH (Cologne, Germany). All spectra were acquired using an NMR spectrometer Avance III 600 (Bruker, Karlsruhe, Germany), magnetic flux density 14.1 T QNP cryo probe head and equipped with automated sample changer Bruker B-ACS 120. Computer Intel Core2 Duo 2.4 GHz with MS Windows XP and Bruker TopSpin 2.1 was used for acquisition, and Bruker TopSpin 2.1 was used for processing.

Determination of Iron Content

Marine PL were digested with 5 mL HNO₃ (65 %) and 150 µL of HCl (37 %) in a microwave oven at 1,400 W (Anto Paar multiwave 3000, Graz, Austria) for 1 h. The samples were further digested with 150 µL H₂O₂ for another 45 min. Thereafter, the iron concentration was measured by an atomic absorption spectrophotometer (AAS 3300, Perkin Elmer, MA, USA). Two digestions were made from each sample and the measurements were performed in duplicate.

Determination of Ethoxyquin, Astaxanthin and Tocopherol

Approximately 0.5 g of marine PL was used for extraction with heptane (5 mL) and the extract was analyzed for astaxanthin, tocopherol and ethoxyquin content by HPLC analysis (Agilent 1100 series, Agilent Technologies, Palo Alto, CA, USA). For determination of tocopherol, a Water Spherisorb (R) silica column (4.6 × 150 mm, i.d. = 3 µm) was used. The mobile phase consisted of heptane and iso-propanol (100:0.4, respectively) and was introduced at a flow rate of 1 mL/min. Tocopherols were detected with a fluorescence (FLD) detector at 290 nm as the excitation wavelength and at 330 nm as the emission wavelength according to the AOCS Official Method Ce 8-89 [13].

For determination of astaxanthin, a LiChrosorb(R) Si60-5 (CP28295, 100 × 3 mm, i.d. = 5 µm) was used. This mobile phase consisted of heptane and iso-propanol (86:14) and was introduced at a flow rate of 1.2 mL/min. Astaxanthin was detected using a DAD detector at 470 nm. For determination of ethoxyquin, the heptane extract was evaporated under nitrogen to dryness and the following residue was redissolved in acetonitrile and analyzed using a C18 Thermo hypersil ODS column (250 mm, i.d. = 4.6 µm). Ethoxyquin was detected with a UV detector at

362 nm according to the method described by He and Ackman [14]. The mobile phase consisted of acetonitrile and 1 mM ammonium acetate (80:20, respectively) and was introduced at a flow rate of 0.8 mL/min. Two extractions were made from each sample and the measurements were performed in duplicate and quantified by authentic standards.

Determination of Peroxide Value (PV) and Free Fatty Acids (FFA) Content

Peroxide value of marine PL was measured by the colorimetric ferric-thiocyanate method at 500 nm using a spectrophotometer (Shimadzu UV-160A, UV-Vis, Struers Chem A/S, DK) as described by IDF (1991) and Shantha and Decker (1994). FFA values of marine PL were determined according to the AOCS method Ce 5a-40 [15]. Both analyses were performed in duplicate.

Preparation of Marine PL Emulsions

Different formulations of marine PL oil-in-water emulsions (300 mL for each formulation) were prepared either with PL alone or with PL and fish oil (Table 1). Emulsions were prepared in two steps: pre-emulsification and homogenization. For the preparation of emulsions comprising both fish oil and marine PL, marine PL in liquid form (MPL, MPT) was first mixed with fish oil whereas marine PL in

Table 1 Experimental design for the emulsions containing marine PL

Formulations/ emulsions	Fish oil (%)	Phospholipids (%)			Total lipids (%)
		MPT	MPL	LC	
MPL2			2.0		2.0
MPL4			4.0		4.0
MPL6			6.0		6.0
MPL8			8.0		8.0
MPL10			10.0		10.0
FMPL05	9.5		0.5		10.0
FMPL1	9.0		1.0		10.0
FMPL2	8.0		2.0		10.0
FMPL3	7.0		3.0		10.0
MPT2		2.0			2.0
MPT10		10.0			10.0
FMPT05	9.5	0.5			10.0
FMPT3	7.0	3.0			10.0
LC2				2.0	2.0
LC10				10.0	10.0
FLC05	9.5			0.5	10.0
FLC3	7.0			3.0	10.0

solid form (LC) was first dissolved in 10 mM acetate-imidazole (pH 7) buffer solution and stirred vigorously overnight at room temperature prior to pre-emulsification with fish oil. In the pre-emulsification step, marine PL or a combination of fish oil and marine PL were added to the buffer over 1 min under vigorous mixing (19,000 rpm) with an Ultra-Turrax (Ystral, Ballrechten-Dottingen, Germany) followed by 2 min of mixing at the same speed. All pre-emulsions were subsequently homogenized in a Panda high pressure table homogenizer (GEA Niro Soavi SPA, Parma, Italy) using a pressure of 800 bar and 80 bar for the first and second stages, respectively. After homogenization, 1 mL of sodium azide (10 %) was added to each emulsion (220 g) to inhibit microbial growth. Emulsions (220 g for each formulation) were stored in 250-mL bottles under two different storage conditions; 2 °C or room temperature (approx. 20–25 °C) in darkness. Samples were analyzed for their physical stability, which include particle size distribution (PSD) and emulsion separation after 0, 4, 8, 16 and 32 days. In order to study the oxidative and hydrolytic stability of marine PL emulsions, FFA and PV were determined before and after 32 days storage. For this, samples were taken, flushed with nitrogen and stored at –40 °C until further analysis.

Determination of Particle Size Distribution

Droplet sizes were determined in the different emulsions using laser diffraction with a Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire, UK). Approximately eight drops of emulsion were suspended directly in recirculating water (3,000 rpm), reaching an obscuration at 15–18 %. The refractive indices of sunflower oil (1.469) and water (1.330) were used as particle and dispersant, respectively. Sampling was made on day 0, 16 and 32 and results are given as surface weighted mean, $D[3,2]$. Other parameters such as volume weighted mean, $D[4,3]$; 10, 50 and 90 % of droplet size which is $d(0.1)$, $d(0.5)$ and $d(0.9)$, respectively were determined as well for multivariate data analysis. The analyses were performed in duplicate.

Measurement of Zeta Potential

The surface charge of the emulsion droplets were determined by the zeta potential with a Zetasizer Nano 2S (Malvern Instruments, Ltd., Worcestershire, UK) at 25 °C. Each sample was diluted in 10 mM sodium acetate–imidazole buffer (pH 7), approximately 20 μ l of the sample in 10 mL buffer before measuring at 25 °C, and the zeta potential range was set to –100 to +50 mV. Results are given as averages of four or more consecutive measurements on the same sample.

Microscopic Examination

An optical light microscope (Olympus BX51, Olympus Co., Tokyo, Japan) was used to observe the structure of the marine PL emulsions. The emulsion samples were smeared on microscope slides and observed at 100 \times magnification (UPL SAPO100XO). Samples were also colored using Nile Red and observed under a fluorescence microscope (Olympus BX51, Olympus Co., Tokyo, Japan) at 100 \times magnification (UPL SAPO100XO). In addition, a cryo-SEM image was prepared to take a closer look at the droplets in marine PL emulsions. For this purpose, emulsions were put into 3-mm aluminium planchettes for high pressure freezing (BAL-TEC, Liechtenstein) without spacer and frozen in an HPM010 instrument (Bal-Tec, Balzers, Liechtenstein). The frozen samples were transferred to a freeze-etching device BAF 060 (Bal-Tec) where they were fractured. The samples were subjected to sublimation by raising the temperature to –95 °C for 5 min and afterwards shadow coated at a 45° angle with 3 nm tungsten. Microscopy was performed at –120 °C in a field emission SEM Leo Gemini 1530 (Carl Zeiss, Oberkochen, Germany) and the imaging was performed with an in-lens detector at 1 kV.

Determination of Emulsion Separation, ES (%)

For each formulation, two test tubes were filled with 10 mL of the emulsion and closed with a cap. Samples were stored at 2 °C or room temperature. The height of the total system and the height of cream separated out at the top were measured on days 1, 2, 6, 10, 16, and 32. Emulsion separation was calculated as: $\text{creaming layer}/\text{total height} \times 100$ %. A larger percentage of the emulsion separation indicated a less stable emulsion. If no cream formation was observed, the emulsion separation was set to 0 %.

Determination of Peroxide Value and Free Fatty Acids Content in Emulsions

Lipids were extracted from the emulsions according to the Bligh & Dyer method [16] using a reduced amount of the chloroform/methanol (1:1 w/w) solvent. Both PV and FFA measurement were carried out on lipid extract according to the methods mentioned previously. Two extractions were made from each sample and the measurements were performed in duplicate for samples before and after 32 days storage.

Statistical Analysis

The PV and FFA data were subjected to one way ANOVA analysis and comparison among samples were performed

with *Bonferroni* multiple comparison test using a statistical package program Graphpad Prism 4 (Graphpad Software Inc., San Diego, USA). Multivariate analysis was performed by the LatentiX 2.0 software package (Latent5 Aps, Frederiksberg, Denmark). The main variance in the data set was studied using principal component analysis (PCA). The data set included variables of physical stability (changes of emulsion volume (EV) after 1 day or 32 days storage and particle size distribution, PSD) and variables for hydrolytic and oxidative stability (PV and FFA at both storage conditions). All data were centered and auto-scaled to equal variance prior to PCA analysis. Significant differences were accepted at ($P < 0.05$).

Results and Discussion

Composition of PL Raw Materials

Peroxide value, FFA, antioxidant and PL content in PL raw materials might affect both the physical and oxidative stability of marine PL emulsions. For this reason, chemical composition of the commercial raw materials was investigated prior to investigation of the marine PL emulsions' stability (Table 2). Among the marine PL, MPT had the highest initial PV and the lowest iron content. On the contrary, MPL had lower initial PV and 10 times higher iron content than MPT. Thus, LC contained the lowest initial amount of hydroperoxides and trace amount of iron and was thus considered to be of higher quality. LC also contained much lower TAG (1 %) and much higher CHO (15 %), and the opposite was the case for both MPT and MPL. In addition, LC also had much higher antioxidant content (mainly α -tocopherol) compared to the other marine PL preparations. Even though other marine PL preparations contained much lower α -tocopherol, they contained additional antioxidants, namely astaxanthin in MPT and ethoxyquin in MPL, respectively. Ethoxyquin is well known as antioxidant and usually used as such in fish meal or fish feed [14].

As far as hydrolytic products were concerned, MPT had much lower FFA and LPC contents compared to the other marine PL preparations indicating the least hydrolysis in MPT. In terms of total PL contents, approximately 30–44 % of PL was found in these three marine PL preparations, with MPT having the lowest PL content. Despite this, MPT had the highest phosphatidylcholine (PC) content and MPL had the lowest. In terms of fatty acid composition, for all the marine PL preparations investigated the PL fraction contained higher EPA and DHA as compared to NL fraction (Table 3). For instance, total EPA and DHA content in PL fraction ranged from 45 to 55 % as compared to 19–37 % in NL fraction. The finding was in agreement with those described in the literature [1, 17].

Physico-chemical Properties of Emulsions

Particle Size Distribution and Zeta Potential

Emulsions solely containing PL (MPT, MPL and LC) had monomodal PSD with average particle diameters ranging from 0.106 to 0.124 μm at 2 °C storage (Fig. 1a; Table 4). According to Mozafari and colleagues [18], the diameters of liposomes range from 20 to 100 nm for small unilamellar vesicles or a diameter >100 nm for large unilamellar vesicles. Hence, the particle size at the peak of 0.1 μm might indicate the presence of liposomes in the marine PL emulsions or small droplets covered by a monolayer of PL. PL can spontaneously self-assemble and form liposomes in the presence of water, and thus they can be formed during homogenization when present in an excess amount. It is therefore likely that such structures were formed during homogenization in addition to the formation of emulsified oil droplets [19, 20]. In addition, other studies [21, 22] have shown that micelles can be formed from monolayers of PL molecules with the hydrophobic fatty acids chains facing the middle. Their average diameter is around 4 nm, which is also the average membrane thickness of a bilayer liposome. However, the lower limit of detection for the MasterSizer 2000 used in this study was 20 nm and therefore the measurement of micelles was impossible. Storage condition (2 °C or room temperature) caused no significant changes in PSD of emulsions solely containing PL even after 32 days (Fig. 1).

Figure 1b shows the PSD for marine PL emulsions that were prepared with different ratios of fish oil and marine PL. Emulsions with the highest proportion of marine PL (FMPL3, FLC3 and FMPT3) had a bimodal PSD with a larger population of smaller droplets and a smaller population of bigger droplets (Fig. 1b, c, d). Smaller droplets (mean diameter at peak 0.1 μm) indicate the presence of PL liposomes whereas bigger droplets (mean diameter at peak 2 μm) indicate the presence of TAG oil droplets surrounded by PL. For two of the emulsions containing the lowest percentage of marine PL (FMPL05 and FLC05), a bimodal PSD with a smaller population of smaller droplets and a larger population of bigger droplets was observed (Fig. 1b, c). The PSD obtained suggested that most of the particles were present as oil droplets surrounded by a PL monolayer in these emulsions. Interestingly, a bimodal PSD was not obtained when MPT was used to prepare a fish oil emulsion with minimum amount of marine PL (FMPT05) as shown in Fig. 1d. This could be due to the lower content of PL in this raw material. In general, Table 4 shows that mean droplet sizes increased with an increase in fish oil concentration and with a decrease in PL concentration. This could also be explained by the shift in the PSD from smaller droplets population to bigger

Table 2 Composition of commercial marine PL used for emulsions preparation

Name Sources	MPT Salmon	MPL Sprat fish meal	LC Fish by products
Peroxide value (mequiv/kg)	3.48 ± 0.51	1.86 ± 0.78	1.75 ± 0.09
Transition metal (Iron, mg/kg)	1.85 ± 0.50	25.75 ± 4.71	2.01 ± 4.57
Triglycerides, TAG (% w/w)	48.0 ± 2.2	40.0 ± 1.5	1.0 ± 0.7
Cholesterol, CHO (% w/w)	5.0 ± 1.5	3.0 ± 1.2	15.0 ± 2.1
α -Tocopherol (mg/kg)	341.1 ± 4.74	94.2 ± 3.74	1464.2 ± 10.84
Astaxanthin (mg/kg)	18.8 ± 0.86	–	–
Ethoxyquin (mg/kg)	–	108.7 ± 8.14	–
Free fatty acids (% w/w)	3.5 ± 0.7	17.0 ± 0.16	21.0 ± 0.23
Total phospholipids (% w/w)	28.43	40.10	43.84
Lysophosphatidylcholine LPC (% w/w)	0.17	2.40	3.47
Phosphatidylcholine PC (% w/w)	24.74	18.90	20.87
Phosphatidylethanolamine PE (% w/w)	3.01	6.00	6.11
Phosphatidylinositol PI (% w/w)	0.51	2.50	0.96
Sphingomyelin SPM (% w/w)	–	–	1.59
Other phospholipids (% w/w)	–	10.30	–

Table 3 Fatty acid compositions of commercial marine phospholipids

Fatty acids composition	Phospholipids [(PL) %]*					
	Neutral lipids fraction			Phospholipids fraction		
	MPT	MPL	LC	MPT	MPL	LC
C14:0	3.08	5.95	4.32	1.42	1.37	2.06
C16:0	8.63	17.19	19.60	14.15	23.96	28.23
C16:1 (n-7)	5.92	6.04	7.79	1.66	2.33	0.46
C16:2 (n-4)	0.36	0.44	3.29	0.23	0.47	0.45
C18:0	1.94	2.26	3.18	6.61	2.15	2.05
C18:1 (n-9)	14.91	16.38	11.63	10.73	11.16	3.22
C18:1 (n-7)	2.52	2.10	0.00	2.57	2.11	0.32
C18:2 (n-6)	1.77	2.14	0.00	0.72	0.92	0.00
C18:3 (n-3)	1.59	1.82	0.00	0.46	0.62	0.00
C18:4 (n-3)	2.29	2.79	0.00	0.48	0.49	0.08
C20:1 (n-9)	1.02	5.20	7.64	2.31	0.43	3.14
C20:4 (n-6)	1.30	0.48	0.00	1.35	1.19	1.81
C20:4 (n-3)	3.37	0.58	0.00	1.35	0.31	0.00
C20:5 (n-3)EPA	17.90	7.95	8.76	15.82	11.50	14.89
C22:1 (n-11)	0.08	7.67	8.83	0.41	0.20	0.00
C22:5 (n-3)	5.60	0.79	0.00	4.36	0.77	0.40
C22:6 (n-3)DHA	18.96	11.14	17.05	29.14	35.42	40.03
C24:1 (n-9)	0.00	1.18	0.00	0.18	1.84	0.00
Others	5.35	4.12	7.93	2.58	1.41	1.46
EPA + DHA	36.87	19.10	25.81	44.95	46.92	54.91
n-3	51.21	26.04	25.81	52.48	49.43	56.11
n-6	3.77	3.12	0.00	2.88	2.40	1.81
n-9	15.93	22.92	19.27	13.21	13.43	6.47
SAFA	14.51	26.61	27.10	23.69	28.22	32.94
MUFA	24.80	32.19	35.88	18.16	18.07	7.24
PUFA	55.34	29.60	29.10	55.58	52.30	58.37
Total	100.00	100.00	100.00	100.00	100.00	100.00

* Values are means ($n = 2$, SD < 5 %)

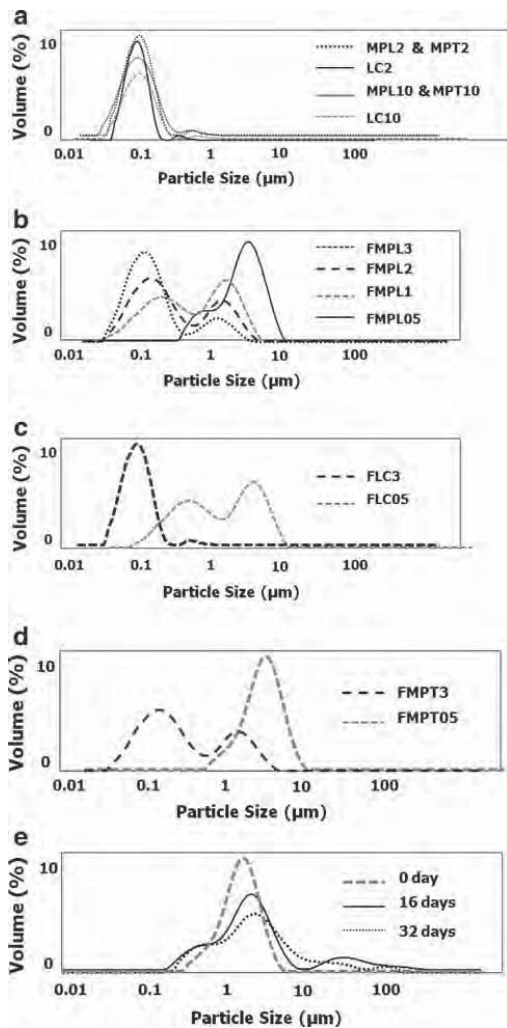


Fig. 1 Particle size distribution of **a** emulsions containing marine PL as the only lipid source, **b–d** emulsions containing mixtures of fish oil and marine PL in different ratios after 32 days storage at 2 °C, **e** FMPT05 after 32 days storage at room temperature

droplets population as shown in Fig. 1b. Similar to the emulsions containing only PL, both storage conditions caused no significant changes in PSD for most of the fish-oil-containing marine PL emulsions except for FMPT05 (Table 4). Storage at room temperature slightly increased and changed the particle size distribution of emulsion FMPT05 (Fig. 1e).

Zeta potential was measured for selected emulsions, namely MPL10, FMPL3, MPT10, FMPT3, LC10 and FLC3. Negative zeta potential was observed in these

emulsions (Table 4). Emulsions that were prepared from MPL and LC had a more negative droplet surface charge (−50 to −60 mV) than emulsions from MPT (−30 to −36 mV). In general, the less negative zeta potential of emulsions containing MPT might explain the finding that particle size distribution changed in FMPT05 at room temperature during storage in contrast to all other emulsions with more negative zeta potential, which showed no changes of PSD even after 32 days storage under both storage conditions.

Microscopy Inspection

Micrographs of emulsion solely containing marine PL, MPL10 (Fig. 2a, b) and emulsion containing both fish oil and marine PL, FMPL05 (Fig. 2c, d) were different. MPL10 contained mainly tiny particles with a few countable bigger particles (Fig. 2b). These tiny particles most likely indicate the presence of liposomes, whereas the bigger droplets most likely indicate the presence of oil droplets surrounded by PL monolayers. The structures of the liposomes were further confirmed by examination using a fluorescence microscope (Fig. 2a), which showed the presence of tiny bright orange spots that indicate the presence of lipid vesicles. The observation from the micrograph was in accordance with that of PSD, which also showed that the emulsion containing solely marine PL had mainly liposomes (as shown by a peak at 0.1 μm in Fig. 1a). In contrast, micrograph of FMPL05 showed that this emulsion contained mainly oil droplets with the particle sizes of 3–5 μm. Furthermore, micrographs from cryo-SEM of FMPL05 (Fig. 2e, f) show the presence of oil droplets with the sizes less than 2 μm in this emulsion. The sizes of the particles in the micrograph were in agreement with that of PSD as shown in Fig. 1b. In addition, a closer look at a large oil droplet in FMPL05 (Fig. 2f) shows the presence of many tiny droplets on the surface of the large droplet. The sizes of these tiny droplets ranged from 50 to 100 nm. This suggests that the large oil droplet was covered by liposomes (small unilamellar vesicles) or tiny PL monolayer-encased oil droplets. The presence of these small structures was not apparent from the PSD (Fig. 1b) probably due to the close association with larger lipid droplets, which made them undetectable by the laser light scattering measurement. It was assumed that the same observation would be obtained for other o/w emulsions containing both fish oil and marine PL namely FMPL1, FMPL2 and FMPL3.

Emulsion Separation (ES) and Physical Appearance

In addition to particle size distribution, the physical stability of emulsions was also investigated by emulsion

Table 4 Mean droplets size and zeta potential of marine PL emulsions after 32 days storage at 2 °C and room temperature, respectively

Formulations	Mean droplets size [D 3, 2] (µm)						Zeta potential (mV)
	Storage at room temperature			Storage at 2 °C			
	0 day	16 days	32 days	0 day	16 days	32 days	
MPL2	0.124	0.124	0.119	0.124	0.111	0.119	
MPL4	0.119	0.120	0.120	0.108	0.119	0.119	
MPL6	0.109	0.106	0.109	0.111	0.109	0.109	
MPL8	0.110	0.111	0.110	0.110	0.110	0.109	
MPL10	0.111	0.107	0.107	0.110	0.110	0.110	-60.1 ± 3.73
FMPL05	2.151	2.147	2.041	2.075	2.088	2.075	
FMPL1	0.321	0.309	0.301	0.318	0.393	0.324	
FMPL2	0.191	0.190	0.193	0.192	0.198	0.190	
FMPL3	0.136	0.135	0.135	0.136	0.136	0.137	-50.5 ± 1.67
MPT2				0.108	0.107	0.107	
MPT10				0.115	0.112	0.114	-31.7 ± 0.89
LC2				0.111	0.109	0.106	
LC10				0.115	0.112	0.112	-50.4 ± 0.26
FMPT05	2.236	2.338	2.220	2.225	2.249	2.304	
FMPT3	0.238	0.239	0.212	0.233	0.235	0.222	-36.2 ± 3.67
FLC05	1.115	1.095	0.909	0.631	0.910	0.892	
FLC3	0.105	0.108	0.108	0.109	0.104	0.105	-52.7 ± 1.55

Values are means ($n = 3$, $SD < 5\%$ for mean droplet sizes)

separation (ES) measurement and their physical appearance (Fig. 3). Creaming occurs in emulsions when the upper part of the emulsions became creamier or when there is a phase separation. Emulsions that were prepared with a combination of both fish oil and marine PL showed a tendency to cream or sediment. In contrast, this did not happen in emulsions containing only PL, irrespective of the PL concentration. During the creaming process in emulsions containing fish oil, emulsions remained opaque at the bottom of the emulsion, while a concentrated cream layer developed at the top of the emulsion (data not shown). In fish oil emulsions containing the lowest level of PL (0.5%), creaming occurred fast and a thick cream layer was formed as early as 1 day after storage, but the cream layer changed very little after a 10-day storage (as shown by FLC05, FMPT05 and FMPL05 in Fig. 3). Emulsion separation (%) of FMPT05 increased noticeably after 32 days of storage under both storage conditions (room temperature and 2 °C). This emulsion also showed phase separation into four layers, namely an oil layer, a cream layer, an emulsion layer and a clear solution layer at room temperature as compared to three layers at 2 °C (data not shown). On the contrary, in emulsions containing more PL (3.0%), and less oil, namely FMPL3 and FMPT3, creaming occurred at a slower rate (Fig. 3b, c). Among the fish-oil-containing marine PL emulsions, FLC3 was the most physically stable. This emulsion showed the least formation of a cream layer over

time (Fig. 3b, c). Regarding storage temperature, it was observed that storage at room temperature caused more creaming in emulsions as compared to storage at 2 °C as exemplified by FMPT05 (Fig. 3b, c).

Hydrolytic and Oxidative Stability of Emulsion

The two main chemical degradation pathways of lipids are oxidation and hydrolysis that can be measured through determination of the PV and FFA, respectively. Figure 4a, b show the comparison of FFA value in marine PL emulsions after 32 days of storage under two different conditions (room temperature and 2 °C). FFA were found in the emulsions even before storage and these FFA originated from the raw materials as shown in Table 2. In addition, results showed that there were no significant differences ($P > 0.05$) in FFA content before and after storage for any of the formulations regardless of the storage conditions indicating that no hydrolysis took place in emulsions during storage as they were prepared with a buffer of pH 7. The result obtained was in agreement with a study by Gritt and colleagues [23], which showed that PL hydrolysis was catalyzed by hydroxyl and hydrogen ions, and thus PL hydrolysis was minimal at pH values near 6.5–7. FFA content increased in fish oil containing emulsions with increasing PL content due to the high FFA content in the marine PL raw materials.

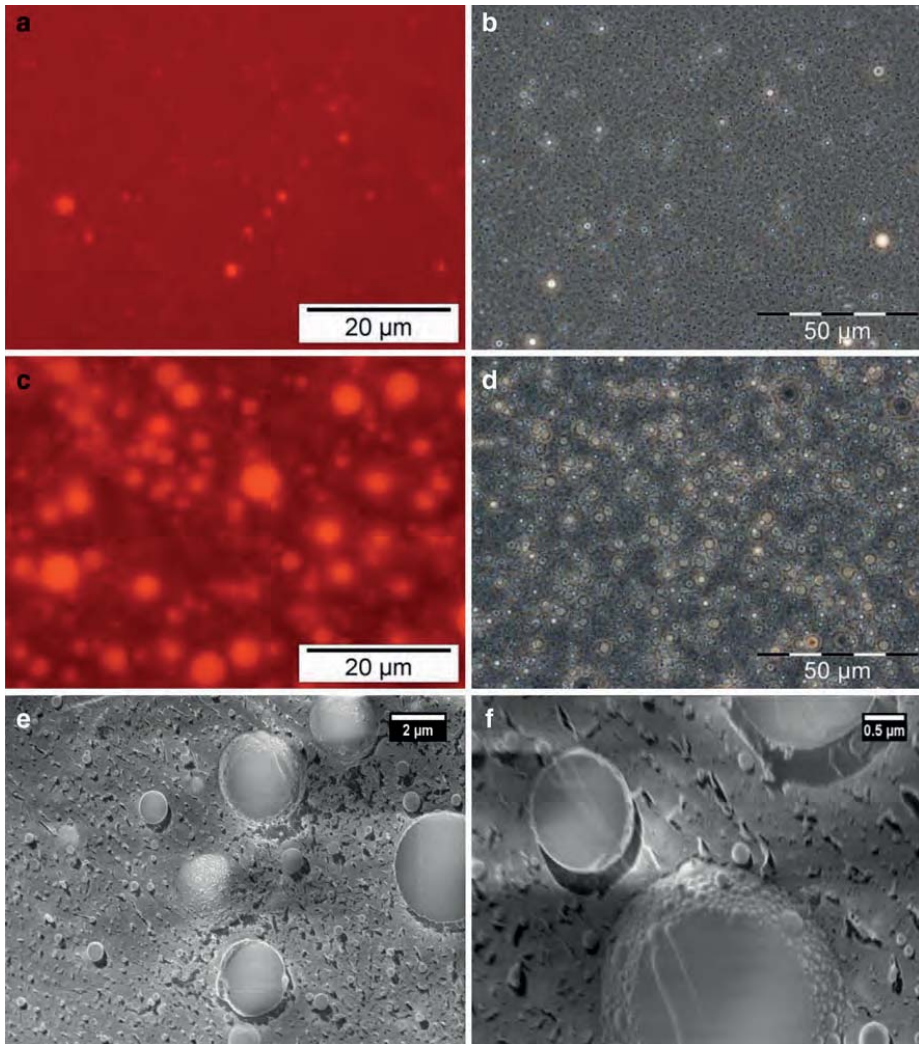
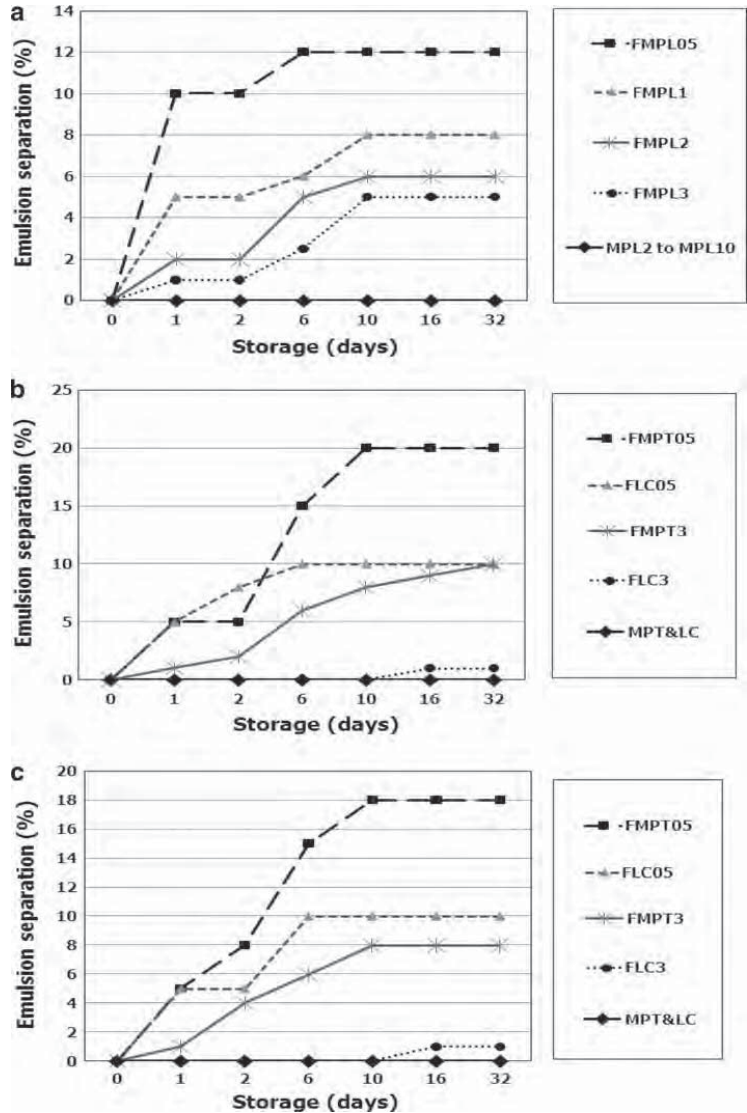


Fig. 2 Micrographs of emulsion MPL10 (a, b); emulsion FMPL05 (c, d) and (e, f). With a, c using fluorescence microscope, b, d using optical microscope and e, f using cryo-SEM

Figure 5a, b show PV of marine PL emulsions before and after 32 days of storage under two different storage conditions. As expected, PV increased more in emulsions that were stored at room temperature. The increase of PV was due to the oxidation of highly unsaturated fatty acids such as EPA and DHA in marine PL or fish oil. As shown in Fig. 5a, smaller PV increment was observed in emulsions containing higher levels of PL. For instance, increasing the PL content in emulsions from MPL2 to MPL10 lowered the PV increment (7 mequiv/kg increment for MPL2 and 3 mequiv/kg increment for MPL10) after

storage at 2 °C. Furthermore, comparison of PV in emulsions MPL10, FMPL05 to FMPL3, showed that the lowest PV increment was in MPL10 whereas the highest PV increment was in FMPL05. All these emulsions contained same level of lipids (10 %) but different levels of PL. The same observation was obtained when comparing emulsions LC10, FLC05 and FLC3. This seems to confirm the antioxidant potential of PL as others have previously reported [24, 25]. Interestingly, MPT/FMPT emulsions behaved differently as shown in Fig. 5b. A high degree of lipid oxidation was observed in these emulsions after 32 days of

Fig. 3 Emulsion separation (%) of marine PL emulsions with and without fish oil after 32 days of storage **a** MPL at 2 °C, **b** MPT and LC at room temperature, **c** MPT and LC at 2 °C. Values are mean \pm SD < 5 % ($n = 2$)



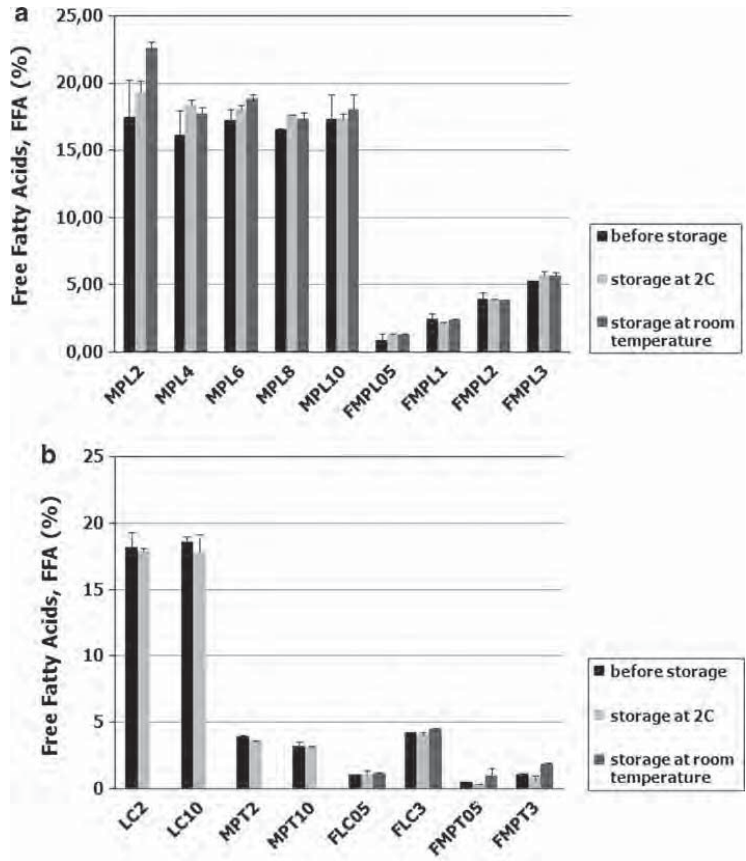
storage and this could be attributed to lower PL content and higher initial content of hydroperoxides in MPT (as shown in Table 2). It is therefore extremely important to use a marine PL raw material of high quality for the production of omega-3 enriched foods.

Multivariate Data Analysis

In order to get an overview of different stability patterns of marine PL emulsions, a PCA was made for emulsions

MPL, FMPL, FMPT and FLC, which were stored at two different storage conditions (room temperature and 2 °C) (Fig. 6). The purpose of this analysis was also to study the relationship between the formulations or chemical composition of marine PL raw materials and the physical and oxidative stability of marine PL emulsions. Emulsions MPT and LC were excluded from the PCA study as they showed no significant difference in mean droplet sizes at any storage condition. Emulsions with higher PL content are located to the left in the plot and emulsions move to the

Fig. 4 Comparison of FFA value of marine PL emulsions before and after 32 days of storage: **a** emulsions from MPL, **b** emulsion from LC and MPT. Values are means \pm SD ($n = 2$)

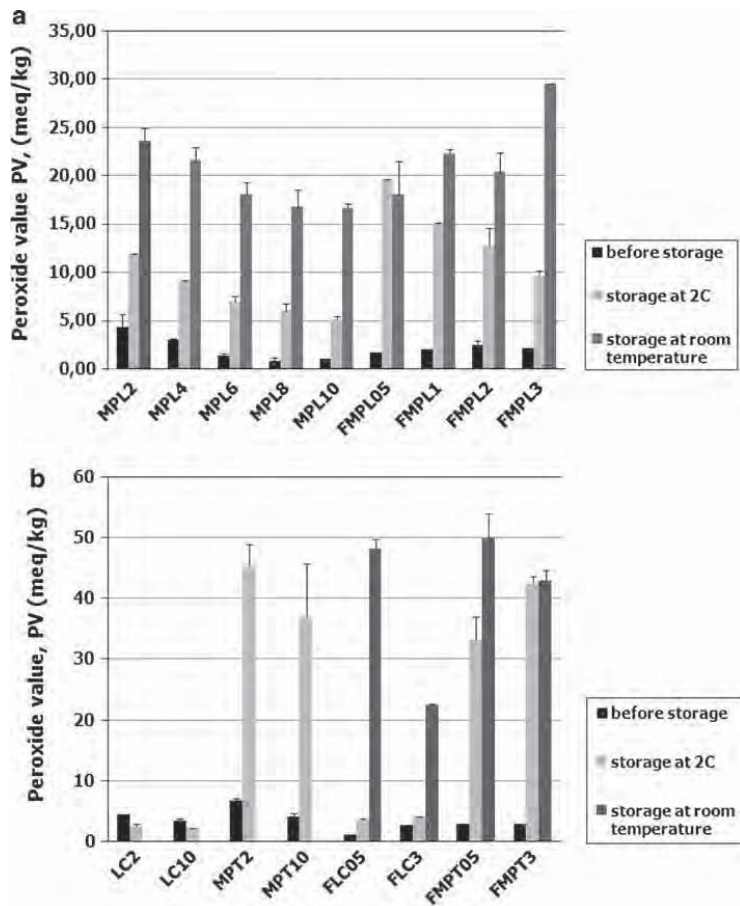


right in the plot with decreasing PL. Hence, emulsions located further to the right had either lower PL: fish oil ratios (e.g. FMPT05 vs. FMPT3), or lower PL content in the emulsifier itself than emulsions located further to the left in the plot (e.g. FLC05 vs. FMPT05). Moreover, all variables of PSD data relating to the physical instability are located to the right in the plot, showing a clear positive correlation between PL content and physical stability, i.e. a low level of PL resulted in the largest droplets.

Emulsions were grouped into three according to stability: group A containing MPL (2–10 %), FMPL (1–3 %) and FLC3; group B containing FLC05 and FMPL05, and group C containing FMPT05 and FMPT3. Group A emulsions are located far from variables of physical stability (ES and PSD) and oxidative stability (PV) indicating that no creaming and less oxidation occurred in these emulsions, respectively and these emulsions that had the best physical and oxidative stability. Emulsions from group

B and C, particularly FMPT05 and FLC05 were physically less stable as they are located near to the variables of PSD. This phenomenon was attributed to their higher fish oil and lower marine PL content. In addition, the discrimination between samples in group B and C is particularly related to the different behavior of FMPT05 emulsion as compared to the other emulsions with low PL, particularly with respect to bigger oil droplets of FMPT05 as shown by the raw data (Table 4). More obvious physical instability was observed in group C emulsions and this was attributed to lower PL content in MPT. Moreover, the higher PV and lower FFA at 2 °C storage after 32 days in group C emulsions also discriminate these emulsions from the other emulsions containing fish oil. Group B emulsions showed the highest degree of physical instability after 1 day of storage, but group C emulsions showed the highest degree of physical instability after 32 days of storage. Moreover, the parameters of changes in PV are also located near group C

Fig. 5 Comparison of FFA values of marine PL emulsions before and after 32 days of storage: **a** emulsions from MPL, **b** emulsion from LC and MPT. Values are means \pm SD ($n = 2$)



emulsions indicating that these emulsions were the least oxidatively stable.

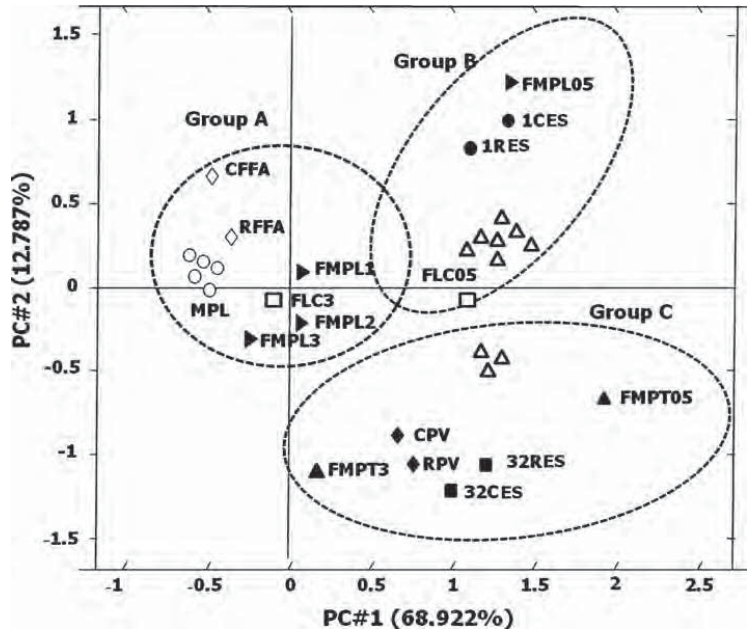
Effect of Physico-chemical Properties of Marine PL on the Physical and Oxidative Stability of Their Emulsions

On the basis of the multivariate analysis as well as the raw data, the relationship between the physico-chemical properties of the marine PL and the resulting physical stability will be discussed in the following. Emulsions containing solely marine PL (MPT, MPL and LC) showed the same good physical stability. The plausible explanations for this phenomenon: (1) the presence of liposomes and micelles as they by nature are thermodynamically stable structures, (2) the negative charge of the monolayer PL at the droplets, which contributed to electrostatic stabilization, (3) the presence of FFA and lysoPL, which most likely contributed

additional charge in addition to that of the PL themselves [26, 27]. It is suggested that FFA increased the negative surface charge of the droplets through their partition into the lipid layer at the o/w interface. Explanations provided in (2) and (3) are supported by the negative zeta potential of the emulsions (Table 4).

Addition of fish oil to the marine PL emulsions decreased their physical stability. FMPT05 was the least physically stable emulsion and this could be attributed to lack of sufficient PL (especially PC content, approximately 0.14 % in FMPT05) to cover the fish oil droplets completely and thus oil droplet aggregation occurred and consequently led to phase separation as suggested by Asai [20]. He reported that the droplet sizes of o/w emulsion prepared from soybean oil (SO) and PC increased drastically and that the emulsion separated into oil and water phases when the PC content was too low (<5 %) to form a PC monolayer that fully covered the oil droplets. Other

Fig. 6 Bi-plot of PCA for both oxidative and physical stability of emulsions: (open circles) MPL; (open squares) FLC; (right sided triangles) FMPL; (filled triangles) FMPT; Variables: (open triangles) parameters of particle size distribution (PSD), changes of emulsion separation (ES) after 1 day (filled circles), and after 32 days (filled squares); (open diamonds) changes of free fatty acids (FFA); (filled diamonds) changes of peroxide value (PV) with R for room temperature and C for storage at 2 °C. Changes of FFA and PV were calculated as differences before and after 32 days storage



factors such as high TAG and low FFA and lysoPC content in the raw material might also have decreased the physical stability as shown in emulsion FMPT05. In contrast, FLC3 showed the best physical stability and this was attributed to the higher content of FFA (21 %), lysoPL (3.47 %), CHO (15 %) and PL (43.84 %) in LC as compared to other marine PL raw materials. In addition, around 21 % of PL in LC is PC, which has a superior emulsifying property in o/w emulsions [10]. It is also speculated that an excessive amount of PL in FLC caused PL monolayer-encased oil droplets to be in equilibrium with PL bilayers, in the form of liposomes, and thus increased the stability of FLC. According to Asai [20], the coexistence of PL monolayer-encased oil droplets and liposomes is crucial to stabilize the o/w emulsion produced with PL as the only emulsifier. In addition, the presence of cholesterol might have increased the rigidity of PL liposomes, their resistance toward degradation and consequently improved the physical stability [23].

As far as the oxidative stability was concerned, emulsions solely containing marine PL or emulsions containing both fish oil and marine PL but with higher content of marine PL showed better stability. This could be attributed to the antioxidative properties of PL that have been found to prevent lipid oxidation regardless of their higher content of EPA and DHA as shown in previous studies [24, 25, 28]. It is also suggested that synergism between PL and α -tocopherol could provide better oxidative stability of

marine PL as shown in many studies [3, 4]. Furthermore, the presence of liposomes might have also given extra oxidative stability to emulsions solely containing marine PL. Some studies [29, 30] showed that marine PL liposomes, namely salmon roe PC liposomes had high oxidative stability and this phenomenon was presumably due to their main molecular species (1-palmitoyl-2-PUFA phosphatidylcholine with PUFA at the *sn*-2 position) that might give a tightly packed molecular conformation. The finding that the smallest increment of PV was found in the LC emulsion can not only be ascribed to the higher content of PL and α -tocopherol in the LC raw material as mentioned earlier, but can also be attributed to the lower content of TAG, higher content of CHO and better quality of the raw material. This issue deserves more attention.

Conclusion

This study showed that the stability of the emulsions depended on their formulations, the quality and the chemical composition of the marine PL used for their preparation and the obtained results thus confirmed our hypothesis. Emulsions containing solely marine PL had good physical stability and could be prepared by using up to 10 % marine PL. The good physical stability of emulsions containing only PL was most likely due to the coexistence of micelles, liposomes and emulsified oil

droplets. However, when formulating physically stable emulsions containing both marine PL and fish oil, there is a requirement for minimum amount of PL to cover fish oil droplets in order to avoid creaming and phase separation. In agreement with other studies, it was found that the minimum amount of PL required to form a stable emulsion was 3 % (equivalent to 0.8–1.3 % of PC depending on the marine PL sources). Regarding oxidation, emulsions with good oxidative stability could be obtained when using raw materials with high purity, less TAG/fish oil content and higher PL, CHO and antioxidant content. In this study, oxidation in marine PL emulsions was evaluated through PV measurement, which shows only the initial stage of lipid oxidation. For this reason, in-depth oxidation studies involving e.g. measurement of secondary volatiles content and sensory evaluation of these types of emulsions should also be performed.

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References

- Peng JL, Larondelle Y, Pham D, Ackman RG, Rollin X (2003) Polysaturated fatty acid profiles of whole body phospholipids and triacylglycerols in anadromous and landlocked Atlantic salmon (*Salmo salar* L.) fry. *Comp Biochem Phys B* 134:335–348
- Wijendran V, Huang MC, Diau GY, Boehm G, Nathanielsz PW, Brenna JT (2002) Efficacy of dietary arachidonic acid provided as triglyceride or phospholipid as substrates for brain arachidonic acid accretion in baboon neonates. *Pediatr Res* 51:265–272
- Cho SY, Joo DS, Choi HG, Nara E, Miyashita K (2001) Oxidative stability of lipids from squid tissues. *Fish Sci* 67:738–743
- Moriya H, Kunimato T, Hosokawa M, Fukunaga K, Nishiyama T, Miyashita K (2007) Oxidative stability of salmon and herring roe lipids and their dietary effect on plasma cholesterol levels of rats. *Fish Sci* 73:668–674
- Henna Lu FS, Nielsen NS, Timm-Heinrich M, Jacobsen C (2011) Oxidative stability of marine phospholipids in the liposomal form and their applications. *Lipids* 46:3–23
- Jacobsen C (2008) Omega-3s in food emulsions: overview and case studies. *Agro Food Ind Hi-Tech* 19:9–12
- McClements DJ (1999) Food emulsions: principles, practice and techniques, 2nd edn. CRC Press, Boca Raton
- Friberg S (1997) Emulsion stability. In: Friberg S, Larsson K (eds) Food emulsions. Marcel Dekker, New York
- Asai Y, Watanabe S (1999) Interaction of sesame oil with soybean phosphatidylcholine and their formation of small dispersed particles. *J Microencapsulation* 16:705–713
- Bueschelberger HG (2004) Lecithin. In: Whitehurst RJ (ed) Emulsifiers in food technology. Blackwell, Oxford, pp 1–39
- AOCS (1998) Official method Ce 2–66: preparation of methyl esters of long chain fatty acids AOCS. Champaign IL, USA
- AOCS (1998) Official method Ce 1b–89: fatty acids composition of marine oils by GLC AOCS. Champaign IL, USA
- AOCS (1998) Official method Ce 8–89: determination of tocopherols and tocotrienols in vegetable oils and fats by HPLC. AOCS, Champaign IL
- He P, Ackman RG (2000) Residues of ethoxyquin and ethoxyquin dimmer in ocean-farmed salmonids determined by high pressure liquid chromatography. *J Food Sci* 65:1312–1314
- AOCS (1998) Official method Ce 5a–40: free fatty acids AOCS. Champaign IL, USA
- Bligh EG, Dyer WJA (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 37:911–917
- Gbogouri GA, Linder M, Fanni J, Parmentier M (2006) Analysis of lipids extracted from salmon (*Salmo salar*) heads by commercial proteolytic enzymes. *Eur J Lipid Sci Technol* 108:766–775
- Mozafari MR, Khosravi-Darani K, Borazan GG, Cui J, Pardakhty A, Yurdugul S (2008) Encapsulation of food ingredients using nanoliposome technology. *Int J Food Prop* 11:833–844
- Rydhag LWI (1981) The function of PL soybean lecithin in emulsion. *J Am Oil Chem Soc* 58:830–837
- Asai Y (2003) Formation of dispersed particles composed of soybean oil and phosphatidyl choline. *Eur J Lipid Sci Technol* 105:397–402
- Thompson AK, Hindmarsh JP, Haisman D, Rades T, Singh H (2006) Comparison of the structure and properties of liposomes prepared from milk fat globule membrane and soy phospholipids. *J Agric Food Chem* 54:3704–3711
- Watwe RM, Bellare JR (1995) Manufacture of liposomes—a review. *Curr Sci* 68:715–724
- Gritt M, Zuidam NJ, Underberg WJM, Crommelin DJA (2011) Hydrolysis of partially saturated egg phosphatidylcholine in aqueous liposome dispersions and the effect of cholesterol incorporation in hydrolysis kinetics. *J Pharm Pharmacol* 45:490–495
- King MF, Boyd LC, Sheldon BW (1992) Effects of phospholipids on lipid oxidation of a salmon oil model system. *J Am Oil Chem Soc* 69:237–242
- King MF, Boyd LC, Sheldon BW (1992) Antioxidant properties of individual phospholipids in a salmon oil model system. *J Am Oil Chem Soc* 69:545–551
- Herman CJ, Groves MJ (1992) Hydrolysis kinetics of phospholipids in thermally stressed intravenous lipid emulsion formulations. *J Pharm Pharmacol* 44:539–542
- Buszello K, Hamisch S, Muller RH, Muller BW (2000) The influence of alkali fatty acids on the properties and the stability of parenteral O/W emulsions modified with Solutol HS 15 (R). *Eur J Pharm Biopharm* 49:143–149
- Boyd LC, Nwosu VC, Young CL, MacMillian L (1998) Monitoring lipid oxidation and antioxidant effects of phospholipids by headspace gas chromatographic analyses of rancimat trapped volatiles. *J Food Lipids* 5:269–282
- Miyashita K, Nara E, Ota T (1994) Comparative-study on the oxidative stability of phosphatidylcholines from Salmon egg and soybean in an aqueous-solution. *Biosci Biotechnol Biochem* 58:1772–1775
- Nara E, Miyashita K, Ota T, Nadachi Y (1998) The oxidative stabilities of polyunsaturated fatty acids in salmon egg phosphatidylcholine liposomes. *Fish Sci* 64:282–286

PAPER III

Lu, F. S. H., Nielsen, N, S., Baron, C. P., & Jacobsen, C.

Oxidative degradation and non-enzymatic browning due to the interaction between oxidized lipids and primary amine groups in different marine phospholipid emulsions.

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Oxidative degradation and non-enzymatic browning due to the interaction between oxidised lipids and primary amine groups in different marine PL emulsions

F.S.H. Lu, N.S. Nielsen, C.P. Baron, C. Jacobsen*

Division of Industrial Food Research, Technical University of Denmark, Søtofts Plads, Building 221, 2800 Kgs. Lyngby, Denmark

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ABSTRACT

Due to the beneficial health effects of marine phospholipids (PL) there is an increasing industrial interest in using them for nutritional applications including emulsified foods. This study was undertaken to investigate both oxidative and hydrolytic stability of marine PL emulsions in relation to the chemical composition of the marine PL used. Moreover, non-enzymatic browning reactions were also investigated. Emulsions were prepared by high pressure homogenizer using different concentrations and sources of marine PL. In some formulations, fish oil was added in order to study the effect of increasing levels of triglycerides in the emulsions. The oxidative and hydrolytic stability of emulsions was investigated through measurement of peroxide value, free fatty acids, and ^{31}P NMR during storage at 2 °C for up to 32 days. The oxidative stability of marine PL emulsions during storage was further investigated through the measurement of secondary volatile compounds by solid-phase microextraction (SPME) and dynamic headspace (DHS) connected to gas chromatography (GC–MS). Non-enzymatic browning reactions were investigated through the measurement of Strecker derived volatiles, colour changes and pyrrole content. The results suggested that the oxidative stability of marine PL emulsions was significantly influenced by the chemical composition and the concentration of marine PL used to prepare them. Emulsions with good oxidative stability could be prepared from marine PL of high purity and high content of PL and antioxidant and low TAG content.

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1. Introduction

Many studies have shown that marine phospholipids PL provide more advantages than marine triglycerides (TAG) available from fish oil. Marine PL have higher content of physiologically important *n*-3 polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) than fish oil (Peng, Larondelle, Pham, Ackman, & Rollin, 2003). EPA and DHA have better bioavailability when provided by PL as compared to TAG (Wijendran et al., 2002). In addition, marine PL have a broad spectrum of health benefits including those from *n*-3 PUFA, their polar head groups and the combination of the two in the same molecule. The health benefits of marine PL have been demonstrated in recent study on krill oil (Ierna, Kerr, Scales, Berge, & Griinari, 2010).

The current knowledge about the oxidative stability of marine PL was recently reviewed by Henna Lu, Nielsen, Timm-Heinrich, and Jacobsen (2011), who reported that several studies have shown that marine PL have better oxidative stability than fish oil regardless of their high degree of unsaturation (Boyd, Nwosu, Young, & MacMillian, 1998). Recent studies have particularly fo-

cused on the oxidative stability of marine PL in liposomal form (Moriya et al., 2007; Mozuraityte, Rustad, & Storro, 2008). It has been suggested that the good oxidative stability of marine PL might be due to (a) their tight intermolecular packing conformation at the sn-2 position (Applegate & Glomset, 1986) and (b) synergism between the phospholipids and α -tocopherol, which is also present in marine PL (Moriya et al., 2007). Furthermore, some studies (Hidalgo, Mercedes Leoan, Nogales, & Zamora, 2007; Hidalgo, Nogales, & Zamora, 2005) showed that slightly oxidised phospholipids in the presence of amino compounds had a better oxidative stability as compared to non-oxidised phospholipids. This was suggested to be due to the formation of antioxidant carbonyl–amine compounds resulting from the reaction between oxidised amino phospholipids/amino acids and fatty acid oxidation products. Similar to the Maillard reaction, the reaction between lipid oxidation products and proteins/PE may result in browning due to formation of pyrroles and both types of reactions are therefore termed as non-enzymatic browning (Zamora, Nogales, & Hidalgo, 2005).

Due to the numerous health benefits of marine PL, there is an increasing desire to use marine PL emulsion as *n*-3 delivery systems with the purpose to increase the *n*-3 PUFA content in foods. A good delivery system is characterised by having a good physical and oxidative stability. To the best of our knowledge, only one study has so far been carried out to investigate the feasibility of

* Corresponding author. Tel.: +45 45252559; fax: +45 45884774.
E-mail address: chja@food.dtu.dk (C. Jacobsen).

marine PL emulsion as delivery system for food enrichment (Lu, Nielsen, Baron, & Jacobsen, in press). However, this study mainly focused on the physicochemical properties of marine PL emulsions and not on their oxidative stability during storage. Therefore, the main objective of this study was to investigate the oxidative stability of marine PL emulsions during storage. We hypothesise that the oxidative stability of marine PL emulsions vary depending on the chemical composition of marine PL used for their preparation. Therefore, the oxidative stability of emulsions prepared with different types of marine PL and with or without addition of fish oil (triglycerides) was investigated. In addition, most of the marine PL that are available in the market are not solely containing PL but also containing residues of amino acids, protein or reducing sugar. The presence of these residues even in small amounts may react with lipid oxidation products in marine PL emulsions as previously mentioned. Therefore, we also measured colour changes, which can be attributed to PL pyrolysis and Strecker derived volatiles, which can be attributed to amino acids degradation in marine PL emulsions.

2. Materials and methods

2.1. Materials

Three different marine phospholipids (LC, MPW and MPL) were obtained from PhosphoTech Laboratoires (Saint-Herblain Cedex, France) and Triple Nine (Esbjerg, Denmark), respectively. Fish oil (Maritex 43-01) was supplied by Maritex (Subsidiary of TINE BA, Sortland, Norway). This fish oil had low initial PV (0.16 meq/kg) and contained 240.4 mg/kg α -tocopherol, 99.3 mg/kg γ -tocopherol and 37.9 mg/kg δ -tocopherol. Sodium acetate and imidazole were obtained from Fluka (Sigma–Aldrich Chemie GmbH, Buchs, Spain) and Merck (Darmstadt, Germany), respectively. All solvents were of HPLC grade (Lab-Scan, Dublin, Ireland).

2.2. Preparation of marine PL emulsion

Different formulations of marine PL emulsion (300 ml for each formulation) were prepared either with PL alone or with PL and fish oil (Table 1). Emulsions were prepared in two steps; pre-emulsification and homogenisation. For the preparation of emulsions comprising both fish oil and marine PL, marine PL in liquid form (MPL, MPW) was first mixed with fish oil whereas marine PL in solid form (LC) was first dissolved in 10 mM acetate-imidazole (pH 7) buffer solution prior to pre-emulsification with fish oil. In the pre-emulsification step, marine PL or a combination of fish oil and marine PL were added to the buffer over 1 min under vigorous mixing (19,000 rpm) with an Ultra-Turrax (Ystral, Ballrechten-Dottingen, Germany) followed by 2 min of mixing at the same speed. All pre-emulsions were subsequently homogenised in a Panda high pressure table homogenizer (GEA Niro Soavi SPA, Parma, Italy) using a pressure of 800 bar and 80 bar for the first and second stages, respectively. After homogenisation, 1 ml of sodium azide

Table 1
Experimental design for marine PL emulsions.

Formulations/ emulsions	%Fish oil	%Phospholipids			%Total lipids	Acetate-imidazole buffer (%)
		MPL	MPW	LC		
MPL		10.0			10.0	90.0
F-MPL	7.0	3.0			10.0	90.0
MPW			10.0		10.0	90.0
F-MPW	7.0		3.0		10.0	90.0
LC				10.0	10.0	90.0
F-LC	7.0			3.0	10.0	90.0

(10%) was added to each emulsion (220 g) to inhibit microbial growth. Emulsions (220 g for each formulation) were stored in 250 ml blue cap bottles at 2 °C in darkness for 32 days. Samples were taken on day 0, 4, 8, 16 and 32, flushed with nitrogen and stored at –40 °C until further analysis. Samples were analysed for their oxidative stability, which included measurement of peroxide value (PV) and measurement of secondary volatiles through Solid Phase Microextraction (SPME) GC–MS (day 16 and 32). In addition to SPME GC–MS analysis, dynamic headspace (DHS) GC–MS analysis was performed on selected samples, namely MPW and F-MPW emulsions (day 16 and 32). In order to study non-enzymatic browning of marine PL emulsion, pyrrole content and colour change (lightness and Yellowness Index, YI) of marine PL emulsions were determined on day 0 and day 32.

2.3. Characterisation of marine phospholipids

2.3.1. Determination of ethoxyquin and tocopherol

Approximately 0.5 g of marine PL was used for extraction with heptane (5 ml) and the extract was analysed for tocopherol and ethoxyquin content by HPLC analysis (Agilent 1100 series, Agilent Technologies, Palo Alto, CA, USA). For determination of tocopherol, a Water Spherisorb (R) silica column (4.6 × 150 mm, i.d. = 3 µm) was used. The mobile phase consisted of heptane and iso-propanol (100:0.4, respectively) and was introduced at a flow rate of 1 ml/min. Tocopherols were detected with a fluorescence (FLD) detector at 290 nm as excitation wavelength and at 330 nm as emission wavelength according to the AOCS Official method Ce 8-89 (1998).

For determination of ethoxyquin, the heptane extract was evaporated under nitrogen to dryness and the obtained residue was redissolved in acetonitrile and analysed using a C18 Thermo hypersil ODS column (250 mm, i.d. = 4.6 µm). Ethoxyquin was detected with a UV detector at 362 nm and the mobile phase consisted of acetonitrile and 1 mM ammonium acetate (80:20, respectively), and was introduced at a flow rate of 0.8 ml/min.

Two extractions were made from each sample and the measurement was performed in duplicate and quantified by authentic standards.

2.3.2. Determination of fatty acid and phospholipids composition

For fatty acids composition in polar lipids and neutral lipids, approximately 0.5 ml marine phospholipids in chloroform (with a concentration of 10–20 mg/ml) was transferred to a Sep-pak column containing 500 mg aminopropyl-modified silica (Waters Corporation, Milford, MA, USA) for lipid separation. A mixture of 2 × 2 ml chloroform and 2-propanol (ratio 2:1) was used to elute the neutral lipid fraction (NL), whereas 3 × 2 ml methanol were used to elute the PL fraction by gravity. Eluates were evaporated under nitrogen and methylated according to AOCS Official method Ce 2-66 (1998), followed by separation through gas chromatography (HP 5890 A, Hewlett Packard, Palo Alto, CA) with a OMEGA-WAX™ 320 column according to the method described by AOCS Official method Ce 1b-89 (1998). The analysis was performed in duplicate. The PL composition of marine PL was determined through ³¹P NMR by Spectra Service GmbH (Cologne, Germany). All spectra were acquired using an NMR spectrometer Avance III 600 (Bruker, Karlsruhe, Germany), magnetic flux density 14.1 Tesla QNP cryo probe head and equipped with automated sample changer Bruker B-ACS 120. Computer Intel Core2 Duo 2.4 GHz with MS Windows XP and Bruker TopSpin 2.1 was used for acquisition, and Bruker TopSpin 2.1 was used for processing.

2.3.3. Determination of lipid classes by thin layer chromatography

The different lipid classes of marine PL were measured by TLC-FID Iatroskan MK-V (Iatron Laboratories, Inc., Tokyo, Japan) with Chromo Star v3.24S software (Bruker-Franzen & SCAP, Germany).

The ten silica gel chromorods SIII (Iatron Laboratories Inc., Tokyo, Japan) were blank scanned twice immediately before sample application in order to remove impurities. Lipids (10–20 mg/ml chloroform-methanol, 2:1) were then spotted on the chromorods using semi-automatic sample spotter (SES GmbH – Analyse system, Germany). The quantification of lipid classes was done by the development in *n*-heptane/diethyl ether/formic acid (70:10:0.02, v/v/v). The neutral lipids (NL) consisting of triglyceride (TAG), free fatty acids (FFA) and cholesterol (CHO) were separated from polar lipids and non-lipid material. After development, the rods were dried in an oven at 120 °C for 2 min and then fully scanned in Iatrosan MK-V. The air and hydrogen flow rates were set at 200 L/min and 160 ml/min, respectively. The scan speed was set at 30 s/rod. The lipid composition of marine PL was expressed as mean percentage of three analyses from each sample.

2.3.4. Determination of iron content

Marine PL were digested with 5 ml HNO₃ (65%) and 150 µl of HCl (37%) in a microwave oven at 1400 W (Anto Paar multiwave 3000, Graz, Austria) for 1 h. The samples were further digested with 150 µl H₂O₂ for another 45 min. Thereafter, the iron concentration was measured by an atomic absorption spectrophotometer (AAS 3300, Perkin Elmer, MA, USA). Two digestions were made from each sample and the measurement was performed in duplicate.

2.3.5. Determination of peroxide value (PV) and free fatty acids (FFA) content

PV was measured on marine PL by the colourimetric ferric-thiocyanate method at 500 nm using a spectrophotometer (Shimadzu UV-160A, UV-vis, Struers Chem A/S, DK) as described by International IDF Standard 74 A (1991) and Shantha and Decker (1994). The FFA values of marine PL were determined according to the AOC Official method Ce 5a-40 (1998) and the measurement was performed in duplicate.

2.3.6. Measurement of pyrrole content

Approximately 0.3 g of marine PL were extracted twice with 6 ml of chloroform-methanol (2:1) with addition of 2 ml of distilled water. The resulting organic and aqueous extracts (methanol-water phase) were analysed for pyrrole content. Organic extract (0.5 g) was dried under nitrogen and 1 ml of 150 mM sodium phosphate (pH 7) containing 3% sodium dodecyl sulphate (SDS) was added. This solution was then treated with Ehrlich reagent (700 µl of reagent A and 170 µl of reagent B). Reagent A was prepared by mixing 2 ml ethanol with 8 ml HCl (2.5 N) while reagent B was prepared by suspending 200 mg of *p*-(dimethylamino) benzaldehyde in 10 ml of reagent A. The final solution was incubated at 45 °C for 30 min. The absorbance of the maximum at 570 nm was measured against a blank prepared under the same conditions but without *p*-(dimethylamino)benzaldehyde. Aqueous extracts (1 ml) was analysed using the same method without further treatment. Two extractions were made from each sample and the measurement was performed in duplicate. Pyrroles content was quantified by an authentic external standard, 1-(4-methoxyphenyl)-1H-pyrrole (this standard give absorbance at 570 nm). The pyrrole concentration is thus given as mM 1-(4-methoxyphenyl)-1H-pyrrole/g emulsion.

2.3.7. Determination of amino acids composition

Approximately of 0.2 g marine PL was extracted with 5 ml of chloroform-methanol (1:1) and was followed by 2.5 ml water. The resulting aqueous extract (methanol-water phase) was analysed for amino acids content by EZ:faast Hydrolysate Amino Acids Analysis kit (Phenomenex, CA, USA). One hundred microlitres of marine PL aqueous extract, 100 µl of internal standard (homoarginine

0.2 mM, methionine-*d*₃ 0.2 mM and homophenylalanine 0.2 mM) were combined in a glass vial and mixed by two short bursts on a vortex. An ion exchange resin solid phase extraction (SPE) tip was attached to a 1.5 ml syringe and the solution was pulled slowly through to completion. Two hundred microlitres of wash solution (water) were added to the glass vial and also pulled slowly through the SPE tip to completion. The 1.5 ml syringe was removed while leaving the SPE tip inside the glass vial. Two hundred microlitres of a premixed elution buffer (sodium hydroxide and *n*-propanol) were then added to the vial. The piston of a 0.6 ml syringe was pulled halfway up the barrel and attached to the SPE tip. Elution buffer was drawn into the SPE resin inside the tip to just before the filter plug and the sorbent material was quickly expelled into the glass vial. This step was repeated until all of the material had been expelled. Fifty microlitres of derivatising reagent (chloroform) was added to the glass vial and the mixture was vortexed vigorously for 8 s. The solution was allowed to react for 1 min and the vortexing step repeated. One hundred microlitres of organic reagent (iso-octane) was then added to the emulsion and vortexed vigorously for 5 s. The mixture was allowed to stand for 1 min for phase separation. After 1 min of the phase separation, 150 µl of the upper organic layer was taken, dried under nitrogen and redissolved with 100 µl of methanol:water (2:1) prior to analysis by LC/MS system (Agilent 1100 series, Agilent Technologies, Palo Alto, CA, USA; column: EZ:faast AAA-MS column 250 × 3.0 mm). The mobile phases consisted of A: 10 mM Ammonium formate in water, B: 10 mM Ammonium formate in methanol and was introduced at a flow rate of 0.5 ml/min. Gradient used: 20 min for 83% B, 20.01 min for 60% B, followed by 26 min for 60% B. The individual compounds were analysed by mass-spectrometry (APCI, positive mode, scan range: 100–600 m/z, APCI ionisation chamber temperature of 450 °C).

2.4. Measurement of lipid oxidation in marine PL emulsions during storage

2.4.1. Determination of peroxide value

Lipids were extracted from the emulsions according to the Bligh and Dyer method using a reduced amount of the chloroform/methanol (1:1 w/w) solvent (Iverson, Lang, & Cooper, 2001). Two extractions were made from each sample and the measurement was performed in duplicate. PV was measured by the colourimetric ferric-thiocyanate method as mentioned earlier using the lipid extract.

2.4.2. Determination of tocopherol content

Lipid extract was weighed (1–2 g) and evaporated under nitrogen prior to analysis by using the same method as mentioned previously. Two extractions were made from each sample and the measurement was performed in duplicate.

2.4.3. Headspace analysis using solid phase microextraction (SPME) GC-MS

Approximately 1 g of emulsion, together with 30 mg of internal standard (10 µg/g of 4-methyl-1-pentanol in rapeseed oil) was mixed on a whirly mixer for 30 s in a 10 ml vial. The sample was equilibrated for 3 min at a temperature of 60 °C, followed by extraction for 45 min at the same temperature while agitating the sample at 500 rpm. Extraction of headspace volatiles was done by 50/30 µm CAR/PDMS SPME fibre (Supelco, Bellefonte, PA, USA) installed on a CTC Combi Pal (CTC Analytics, Waldbronn, Germany). Volatiles were desorbed in the injection port of gas chromatograph (HP 6890 Series, Hewlett Packard, Palo Alto, CA, USA; Column: DB-1701, 30 m × 0.25 mm × 1.0 µm; J&W Scientific, CA, USA) for 60 s at 220 °C. The oven program had an initial temperature of 35 °C for 3 min, with increment of 3.0 °C/min to 140 °C,

then increment of 5.0 °C/min to 170 °C and increment of 10.0 °C/min to 240 °C, where the temperature was held for 8 min. The individual compounds were analysed by mass-spectrometry (HP 5973 inert mass-selective detector, Agilent Technologies, USA; Electron ionisation mode, 70 eV, mass to charge ratio scan between 30 and 250). In order to investigate lipid oxidation in marine PL emulsions, the following secondary volatiles were selected for quantification: pentanal, hexanal and 1-pentanol as volatiles derived from the oxidation of *n*-6 PUFA; octanal and nonanal as volatiles derived from oxidation of *n*-9 MUFA; *E*-2-hexenal, 1-penten-3-one, *Z*-4-heptenal, *E,E*-2,4-heptadienal, *E,Z*-2,6-nonadienal, 2-ethylfuran and propanal as volatiles derived from oxidation of *n*-3 PUFA. Calibration curves were made by dissolving the different volatile standards in rapeseed oil followed by dilution to obtain different concentrations (0.1–10 µg/g). Due to the different retention capacity of volatiles in emulsions with different formulations/matrices, two sets of calibration curves were prepared; a matrix of an emulsion solely containing marine PL and a matrix of an emulsion containing both fish oil and marine PL. In this study calibration curves were parallel shifted in order to obtain positive values. The given values (in ng/g) of the volatiles are thus not the “real” values and should therefore not be used for comparison to other studies. Measurements were made in triplicates on each emulsion. SPME GC-MS analysis was also used for the identification of volatile Strecker degradation products. These volatiles were not quantified through calibration curves. In contrast, abundance values obtained from the MS analysis were used for quantification.

2.4.4. Headspace analysis using dynamic headspace (DHS) GC-MS analysis

Volatiles from 4 g of the selected emulsions were collected by purging the emulsion with nitrogen (150 ml/min) for 30 min at 45 °C, using 4-methyl-1-pentanol as the internal standard, and trapped on Tenax GR tubes (Perkin-Elmer, CN, USA) packed with 225 mg Tenax GR (60–80 mesh, Varian, Middelburg, Netherlands). The volatiles were desorbed (200 °C) from the trap in an automatic thermal desorber (ATD-400, Perkin-Elmer, Norwalk, CT) and cryofocused on a Tenax GR cold trap. The volatiles were separated by gas chromatography (HP 5890 IIA, Hewlett–Packard, Palo Alto, CA) as described by Timm-Heinrich, Xuebing, Nielsen, and Jacobsen (2003) and analysed by mass spectrometry (HP 5972 mass selective detector). The oven temperature program was: 45 °C held for 5 min, 1.5 °C/min to 55 °C, 2.5 °C/min to 90 °C, 12 °C/min to 220 °C and finally held at 220 °C for 4 min. The individual compounds were identified by both MS-library searches (Wiley 138K, John Wiley and Sons, Hewlett–Packard) and by authentic external standards. Calibration curves were made by dissolving the different volatile standards in ethanol followed by dilution to obtain different concentrations (0.01–1 mg/g). The individual compounds were quantified through calibration curves made by adding 1 µl of standards to Tenax GR tubes directly. The same external standards as mentioned earlier were used for quantification of volatile oxidation products.

2.5. Determination of non-enzymatic browning

2.5.1. Measurement of pyrrole content and colour changes

Emulsion sample (3 ml) was extracted twice with 6 ml of chloroform–methanol (2:1) and the resulting organic and aqueous extracts were analysed for pyrrole content and colour changes. The pyrrole content in both organic and aqueous layers was measured according to the method described earlier. Colour changes were only measured on the organic extract, using a spectrophotometer (X-Rite, X-Rite, Inc. Grandville, MI, USA). The instrument was calibrated before each measurement and the results were recorded using the CIE colour system profile of L^* (Lightness), a^* (redness/

greenness), b^* (yellowness/blueness). In addition, yellowness index (YI) was calculated according to Francis and Clydesdale (1975): $YI = 142.86 b^*/L^*$. Two extractions were performed on each sample and the measurement was performed in duplicate.

2.6. Statistical analysis

The obtained data, PV, FFA, colour and pyrrole measurement were subjected to one way ANOVA analysis and comparison among samples were performed with *Bonferroni* multiple comparison test using a statistical package program Graphpad Prism 4 (Graphpad Software Inc., San Diego, USA). Significant differences were accepted at ($p < 0.05$).

3. Results and discussion

3.1. Chemical composition of marine PL

Different initial PV, volatile oxidation products, FFA, antioxidant and iron were present in fish meal and thus also present in marine PL as they were co-extracted. Their presence may affect the stability of marine PL emulsions differently. For this reason, the chemical composition of these raw materials was investigated prior to further discussion of marine PL emulsions' stability. The initial PV of MPW was lower than that of MPL and LC (Table 2). However, the PV data were contradictory to the volatile data, in which the concentration of initial *n*-3 derived volatiles in MPW (64.2 mg/kg) was approximately double of that in MPL (33.4 mg/kg) and LC (25.3 mg/kg) (Table 2). The findings for MPW could indicate that some of the lipid hydroperoxides have been decomposed to secondary volatiles. Taken together, PV and volatiles showed that

Table 2
Composition of marine PL used for emulsions preparation.

Name	MPL	MPW ^a	LC			
Sources	sprat fish meal	sprat fish meal	Fish by products			
Total phospholipids (%)	40.10	41.50	43.84			
Phosphatidylcholine PC (%)	18.90	18.30	20.87			
Phosphatidylethanolamine PE (%)	6.00	4.70	6.11			
Phosphatidylinositol PI (%)	2.50	2.10	0.96			
Sphingomyelin SPM (%)	-	-	1.59			
Lysophosphatidylcholine LPC (%)	2.40	3.40	3.47			
Other phospholipids	10.30	8.90	-			
Triglycerides, TAG (%)	40.0	40.0	1.0			
Cholesterol, CHO (%)	3.0	2.0	15.0			
Free fatty acids, FFA (%)	17.0	16.0	21.0			
% Fatty acids composition (NL-Neutral lipid fraction/ PL-Phospholipids fraction)	NL	PL	NL	PL	NL	PL
<i>n</i> -3	26.04	49.43	26.16	46.76	25.81	56.11
<i>n</i> -6	3.12	2.40	4.82	2.93	0.00	1.81
<i>n</i> -9	22.92	13.43	24.36	16.07	19.27	6.47
SAFA	26.61	28.22	26.71	31.5	27.10	32.94
MUFA	32.19	18.07	39.05	17.92	35.88	7.24
PUFA	26.60	52.30	31.27	50.09	29.10	58.31
EPA +DHA	19.10	46.92	20.45	45.32	25.81	54.91
α -Tocopherol (µg/g)		94.2		73.4		1464.2
Transition metal, iron (ppm)		25.75		20.08		2.01
Peroxide Value (meq/kg)		1.86±0.78		0.81±0.04		1.75±0.09
Initial <i>n</i> -3 derived volatiles (mg/kg)		33.4		64.2		25.3
Pyrrole content (mMol/g marine PL)						
Hydrophobic		9.88±0.52		10.32±0.86		1.60±0.08
Hydrophilic		0.18±0.01		0.37±0.04		0.23±0.01

^a MPL also contained 108.7 mg/kg ethoxyquin.

the MPW raw material was the most oxidised, followed by MPL and LC. Both MPW and MPL were extracted from fish meal at high temperature and this might be the cause of lipid oxidation whereas LC was extracted from fish by-product through enzymatic hydrolysis at lower temperature. Currently, there is no refining process carried out to reduce the colour and volatiles of marine PL as this process might destroy the properties of marine PL. In addition to its lowest degree of oxidation, LC also contained less iron than the other PL preparations and was thus considered to be of better quality (Table 2). In terms of PL contents, 40–44% of PL were found in these three marine PL preparations, with slightly higher total PL and phosphatidylcholine (PC) contents in LC (Table 2). Marine PL used in this study also contained different levels of other lipids such as cholesterol (CHO) and triglycerides (TAG). Thus, LC contained much lower TAG (1%) and much higher CHO (15%) than MPW and MPL, which had approximately the same content of these lipids. In addition, LC also contained residues of amino acids (Table 3) as its total lipid content was approximately 80%, compared to 100% for both MPL and MPW.

FFA and lysophosphatidylcholine (LPC) contents were similar in the three marine PL preparations indicating the same degree of hydrolysis in the marine PL during their manufacturing process (Table 2). In terms of the fatty acid composition of the marine PL preparations, the PL fraction contained higher EPA and DHA as compared to the NL fraction. The total EPA and DHA content in the PL fraction ranged from 45% to 55% as compared to 19% to 26% in the NL fraction. The composition was in agreement with results from other studies (Peng et al., 2003). In general, MPL and MPW had the same lipid and fatty acid composition, the only difference between these two marine PLs was their antioxidant content. MPL contained ethoxyquin in addition to α -tocopherol,

whereas MPW and LC only contained tocopherol, which is naturally present in marine PL (Table 2). Ethoxyquin is usually used as antioxidant in fish meal or fish feed and the ethoxyquin present in MPL had thus been co-extracted together with the lipids from the fish meal. On the other hand, LC had at least 15 times higher α -tocopherol level as compared to both MPL and MPW.

The highest hydrophobic pyrrole content was found in raw material MPW, followed by MPL and LC. MPW also had the highest content of hydrophilic pyrrole content, but in all three PL preparations the content of hydrophilic pyrroles was much lower than the content of hydrophobic pyrroles (Table 2). A high pyrrole content in marine PL might indicate high non-enzymatic browning reaction of marine PL during their manufacturing process.

3.2. Lipid oxidation

3.2.1. Peroxide value

Emulsions solely containing marine PL showed significantly lower ($p < 0.05$) PV increment during storage than emulsions containing both marine PL and fish oil (Fig. 1a). MPL showed higher PV increment after 32 days storage as compared to MPW and LC. PV did not increase in any of the emulsions during the first 4 days of storage. However, PV increased in all emulsions after 8 days of storage except for LC and F-LC, which seemed to be the most stable emulsions with regard to PV development. The PV data confirmed the results obtained in a preliminary experiment on MPL, F-MPL, LC and F-LC. In summary, both storage and chemical composition of marine PL significantly ($p < 0.05$) affected the PV increment of emulsions. However, the interpretation of oxidative stability of marine PL emulsions cannot be made only based on PV measure-

Table 3
Strecker derived volatiles detected by SPME GC–MS in emulsions on day 16, day 32 and list of amino acids residues in raw materials marine phospholipids.

Main volatile compounds	Chromatographic areas (AU) $\times 10^5$ through SPME					
	MPL		MPW		LC [*]	
	Day 16	Day 32	Day 16	Day 32	Day 16	Day 32
<i>Strecker degradation (SD)</i>						
2-Methyl-2-pentenal	–	–	–	–	837	766
Dimethyldisulphide	22.1	17.5	4.7	543	776	775
3-Methylbutanal	19	38.3	15.2	334	199	282
Benzaldehyde	32.9	32.4	37.7	65.8	219	278
Dimethyltrisulphide	6.2	5.2	2.4	42.2	178	194
Pyridines	6.2	5.2	2.4	42.2	178	194
2-Methylpropanal	8.3	6.1	6.9	15.8	17.2	24.1
2-Methylbutanal	3.4	7.6	2.2	21.2	11.2	15.8
Marine PL raw materials % (g/100g marine PL)		MPL		MPW		LC
<i>Amino acids residues</i>						
Leucine		0.01 \pm 0.00		–		–
Proline		–		–		3.49 \pm 0.40
Alanine		0.09 \pm 0.01		0.13 \pm 0.01		4.94 \pm 0.12
Glycine		0.04 \pm 0.00		0.03 \pm 0.00		1.04 \pm 0.36
Glutamic acid		0.02 \pm 0.00		–		0.16 \pm 0.07
Isoleucine		0.01 \pm 0.00		0.01 \pm 0.00		0.14 \pm 0.06
Valine		0.03 \pm 0.00		0.02 \pm 0.00		0.70 \pm 0.07
Phenylalanine		–		–		0.14 \pm 0.06
Arginine		–		–		1.59 \pm 0.30
Lysine		–		–		–
Hydroxyproline		–		–		0.03 \pm 0.01
Histidine		–		–		0.02 \pm 0.00
Tyrosine		–		–		–
Tryptophan		–		–		1.08 \pm 0.17
Serine		0.02 \pm 0.00		0.02 \pm 0.00		0.19 \pm 0.02
Aspartic acid		0.01 \pm 0.00		0.01 \pm 0.00		0.07 \pm 0.02
Threonine		0.02 \pm 0.00		0.02 \pm 0.00		0.06 \pm 0.03
Methionine		–		–		0.04 \pm 0.04
Cysteine		–		–		–
Total		0.26 \pm 0.03		0.25 \pm 0.02		14.23 \pm 0.09

^{*} Trimethylpyrazine, 3-ethyl-2,5-diethylpyrazine and 2-pentylfuran were also found in LC.

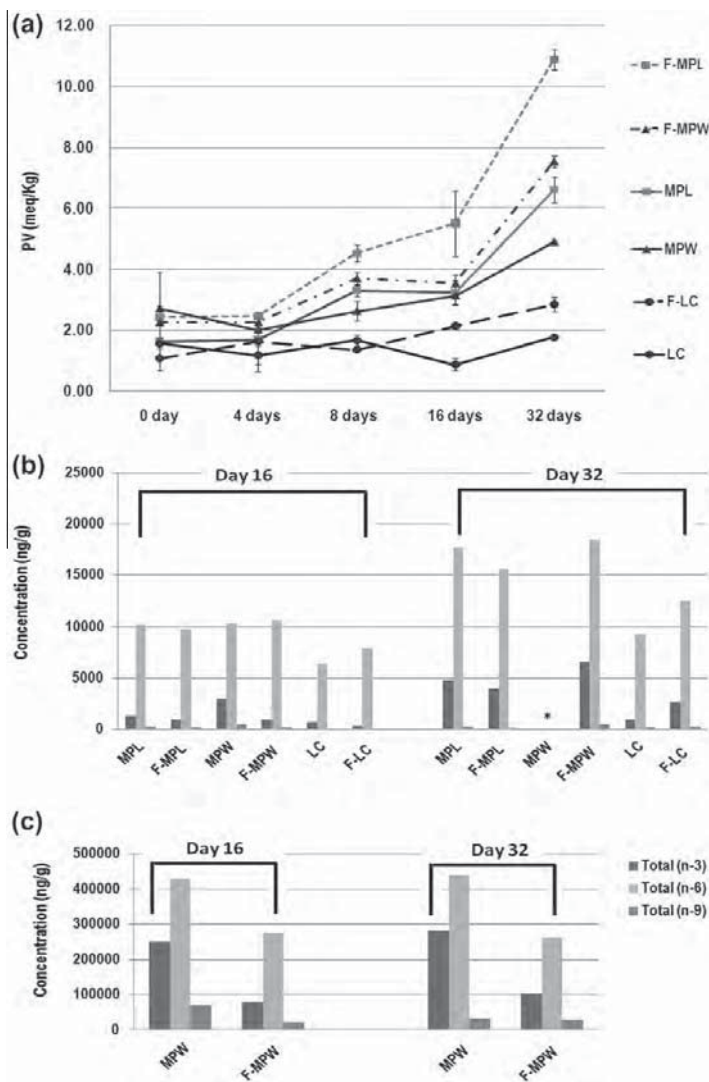


Fig. 1. Oxidative stability of marine PL emulsions upon 32 days storage at 2 °C assessed by (a) formation of peroxide values expressed as meq/kg; (b) volatile oxidation products expressed as the sum of the compounds in ng/g emulsion detected using SPME GC–MS and (c) volatile oxidation products expressed as the sum of the compounds in ng/g emulsion detected using DHS GC–MS. *Missing data Total n-3 includes E-2-hexenal, 1-penten-3-one, Z-4-heptenal, E,E-2,4-heptadienal, E,Z-2,6-nonadienal, 2-ethylfuran and propanal; total n-6 includes pentanal, hexanal and 1-pentanol; total n-9 includes octanal and nonanal.

ment without taking into consideration the secondary volatile oxidation products data.

3.2.2. Secondary lipid oxidation products: volatiles

In order to further study the oxidative degradation in marine PL emulsions, secondary volatile oxidation products were measured by SPME GC–MS in all marine PL emulsions after 16 days and 32 days storage at 2 °C (Fig. 1b). For the MPW emulsion, SPME data showed a large increment of 3-methylbutanal and dimethylsulphide concentrations (Table 3) and a concomitant drastic decrease of other volatiles after 32 days storage (Lu, Nielsen, & Jacobsen, submitted for publication). These findings might be explained by

CAR/PDMS fibres having a greater affinity for low molecular weight volatiles. Thus, volatiles competed for the same binding sites on the CAR/PDMS fibre and it seemed that volatiles with low molecular weight, namely 3-methylbutanal had displaced those with high molecular weight and this consequently led to fibre saturation and unreliable results for the MPW emulsion after 32 days of storage. Therefore, in addition to SPME GC–MS, DHS GC–MS analysis was carried out on these two samples (Fig. 1c).

Taken together Fig. 1b and c showed that in general, the oxidative stability of marine PL emulsion was in the order: MPW < MPL < F-MPW/F-MPL < LC < F-LC after 16 days storage and MPW < F-MPW < MPL < F-MPL < F-LC < LC after 32 days storage. The obtained

result was to some extent contradictory to the PV measurement. Thus, in contrast to the findings for PV, MPW and MPL emulsions solely containing marine PL had higher concentrations of volatile oxidation products than the corresponding emulsions containing both fish oil and marine PL. For instance, MPL had higher level of total secondary volatile compounds degraded from *n*-3 fatty acids (4824 ng/g) than F-MPL (4011 ng/g) after 32 days storage. The higher oxidative stability of emulsions containing both fish oil and marine phospholipids (F-MPW and F-MPL) as compared to the corresponding emulsions without fish oil might be related to their antioxidant content. As far as the tocopherol content is concerned, F-MPW and F-MPL had higher α -tocopherol concentrations (20 mg/kg emulsion) than MPW and MPL (7 mg/kg emulsion). This was due to the high content of tocopherol in fish oil as compared to the tocopherol level in the marine PL preparations used for producing these emulsions. Many studies have shown that PL itself has a protective effect against oxidation but that this protective effect was greatly influenced by the presence of α -tocopherol. It has been suggested that the synergistic effect between PL and α -tocopherol was the main factor responsible for the oxidative stability of marine PL (Moriya et al., 2007). In addition, F-MPW and F-MPL emulsions also contained γ -tocopherol and δ -tocopherol from fish oil. Several studies have shown that γ -tocopherol was a better antioxidant in fish oil enriched food emulsions than α -tocopherol (Jacobsen, 2008). Hence, the presence of γ -tocopherol in emulsions with fish oil could have increased their oxidative stability.

As mentioned above the results also showed that higher concentrations of secondary volatile oxidation compounds was found in MPW and F-MPW emulsions as compared to MPL and F-MPL emulsions after 16 days and 32 days storage. For MPW and F-MPW emulsions, a comparison was also made between both emulsions on day 32 using DHS data as the SPME data at this time point was unreliable as previously mentioned (Fig. 1c). The higher oxidative stability of MPL emulsion as compared to MPW emulsion might be due to the presence of additional antioxidant (108.7 mg/kg of ethoxyquin).

LC had lower concentration of total volatile oxidation compounds (922 ng/g from *n*-3) as compared to F-LC (2717 ng/g from *n*-3) after 32 days storage and a similar result was obtained after 16 days of storage. Thus, LC emulsions behaved differently than MPW and MPL emulsions. This different behaviour might be due to the fact the LC raw material contained much higher levels of tocopherol than the MPL and MPW raw materials. In contrast to the MPL and MPW emulsions, the LC emulsion therefore had a higher content of total tocopherol (130 mg/kg) than the corresponding emulsions with fish oil (63 mg/kg). Moreover, the higher content of PL in the LC emulsion most likely also contributed to its better oxidative stability as PL has been shown to have antioxidative effect against oxidation (Boyd et al., 1998). Furthermore, emulsions based on LC had the lowest level of all types of volatile oxidation products after 16 and 32 days of storage. This phenomenon was partly due to the non-enzymatic browning reactions (reaction between lipid derived volatiles and primary amine group), which thus subsequently reduced the levels of lipid derived volatiles. Taken together, both PV and volatiles data showed that LC was the best raw material to prepare oxidatively stable emulsion. The higher oxidative stability of these emulsions as compared to emulsions based on MPW and MPL can at least partly be attributed to its better chemical composition with a higher content of PC (20.87%), cholesterol (15%), α -tocopherol (1464 mg/kg) and lower content of triglyceride (around 1%) when compared to the other raw materials used in our study.

3.3. Investigation of non-enzymatic browning development

3.3.1. Strecker degradation (SD) volatiles

Strecker degradation of amino acids is a minor pathway in non-enzymatic browning and involves the oxidative deamination of α -

amino acids in the presence of compounds such as reducing sugars or some lipid oxidation products. When the reaction only involves amino acids and reducing sugars it is termed Maillard reaction. The main SD products in MPW, MPL and LC emulsions found from SPME GC–MS determination were 2-methyl-2-pentenal, dimethylsulphide, 3-methylbutanal, benzaldehyde, dimethyltrisulphide, pyridine, 2-methylpropanal and 2-methylbutanal (Table 3). In addition to these volatiles, trimethylpyrazine, 3-ethyl-2,5-diethylpyrazine and 2-pentylfuran were found in marine PL emulsions through (DHS) GC–MS determination. To the best of our knowledge, this is the first study to report the generation of Strecker derived volatile compounds in marine PL emulsions. SD volatiles such as 2-methyl-2-pentenal, benzaldehyde and sulphur containing compounds such as dimethylsulphide and dimethyltrisulphide have been reported by Linder and Ackman (2002) in adductor muscle of the sea scallop *Placopecten magellanicus* (contains 95% PL) using SPME with PDMS and PDMS/DVB fibres. These volatiles have also been reported in products such as shrimp, oyster and anchovy (Chung, Yung, & Kim, 2001). 2-Methyl-2-pentenal was suggested to be the major volatile product from the reaction of the tertiary lipid oxidation product (*E*)-2-(*E*)-4-heptadienal with lysine (Zamora, Rios, & Hidalgo, 1994). 3-Methylbutanal was suggested to originate from the reaction between aldehydic lipid oxidation products with leucine, whereas dimethylsulphide and dimethyltrisulphide were found to be the degradation products of methionine (Ventanas, Estevez, & Delgado, 2007). The low content of leucine, lysine and methionine in marine PL (as shown in Table 3) confirmed that these amino acids were already degraded to form Strecker aldehydes in marine PL emulsions during storage.

The results show that there were higher concentrations of SD products in LC emulsions, followed by MPW and MPL emulsions, which had similar levels (Table 3). This could be due to the high content of amino acid residues in the LC raw material as previously discussed. Most of the SD volatiles, namely 2-methyl-2-pentenal, dimethylsulphide, dimethyltrisulphide were detected in LC emulsion even before the storage and the concentrations of these volatiles remained constant after 16 and 32 days of storage, except the slight increase of benzaldehyde, 2-methylpropanal and 3-methylbutanal (Table 3).

It is suggested that these Strecker aldehydes were produced from amino acid residues present in the marine PL preparations and via the reaction with tertiary lipid oxidation products such as unsaturated epoxy keto fatty esters, epoxyalkenals and hydroxyalkenals, as shown in Fig. 3. It has been proposed that the presence of two oxygenated function groups in the tertiary lipid oxidation products, namely one carbonyl group and one epoxy or hydroxyl group is required for the SD reaction to occur as shown in mechanism A in Fig. 3 (Hidalgo & Zamora, 2004; Zamora, Gallardo, & Hidalgo, 2007). In addition, according to Zamora et al. (2007), secondary lipid oxidation products such as alkadienals and ketodienes also can degrade the amino acids to their corresponding Strecker aldehydes under appropriate conditions when they undergo further oxidation. It is speculated that most of the SD reaction occurred in marine PL during their manufacturing process since their level did not seem to change significantly during storage of our PL emulsions. This may be because marine PL (for both MPW and MPL) were extracted from fish meal at high temperature and this caused lipid oxidation and led to the generation of secondary and tertiary lipid oxidation products. Lipid oxidation of *n*-3 fatty acids amongst other produces 2,4-heptadienal (secondary oxidation product), which subsequently form 4,5 (*E*)-epoxy-2-(*E*) heptenal with two oxygenated function groups (tertiary lipid oxidation products). The concentrations of most of the SD volatiles remained constant or slightly increased in MPL and MPW emulsions throughout storage (except the increase of 3-methylbutanal in MPL, and the increase of both 3-methylbutanal and dimethyl-

disulfide in MPW emulsion after 32 days storage) as shown in Table 3. These findings might indicate that SD reactions occurred in marine PL emulsions during storage in parallel to the lipid oxidation reaction. It is possible that the increase of SD volatiles could have enhanced lipid oxidation in MPW and F-MPW emulsions or vice versa (as shown by the increase of volatile oxidation products in these emulsions). However, no clear conclusion could be made about the exact reaction/interaction between lipid oxidation and Strecker degradation in marine PL emulsions in this study and further studies are required to elucidate such interactions.

3.3.2. Pyrrolisation

Non-enzymatic browning reactions produced a large variety of chemical structures, including both volatile and non-volatile compounds. The non-volatile compounds included pyrroles, which have a heterocyclic structure. There was no significant change ($p > 0.05$) of the pyrroles content in any of the emulsions during 32 days storage at 2 °C (Fig. 2a). However, this did not necessarily indicate an absence of non-enzymatic browning development in marine PL emulsions during storage as a slight increase of SD products was observed in marine PL emulsions after storage as mentioned earlier. Low amounts of both hydrophilic and of hydrophobic pyrroles were found in LC and F-LC emulsions after both 0 and 32 days of storage. In contrast, much higher concentrations of hydrophobic pyrroles were found in both MPL and MPW emulsions with and without addition of fish oil, although the pyrroles content was lower in emulsions with fish oil due to the dilution by fish oil. The pyrrole content in emulsions correlated with the pyrrole content in the raw materials. The highest content of both hydrophobic and hydrophilic pyrroles was thus found in raw material MPW, followed by MPL and LC (Table 2). The lower pyrrole content in MPL emulsion as compared to MPW emulsion might be due to additional protection of MPL raw material by ethoxyquin during its manufacturing process. The high hydrophobic pyrrole content in MPW and MPL raw materials therefore suggested that non-enzymatic browning development in the raw materials of MPW and MPL occurred during their manufacturing

process as also suggested for the SD products. In addition, pyrroles could also be formed through protein pyrrolisation by lipid oxidation products during the storage of fish and during the fish meal production at lower temperature. According to Hidalgo, Alaiz, and Zamora (1999), protein pyrrolisation with lipid oxidation products occur rapidly at 25–50 °C and exhibited high colour changes and amino losses in the model study they carried out with bovine serum albumin and hydroperoxides and secondary products of methyl linoleate oxidation.

In the present study, non-enzymatic browning may originate from the reaction between reactive carbonyls, such as lipid oxidation products, with the amino group from phosphatidylethanolamine (PE) or amino acids residues present in marine PL (Fig. 3). Besides the tertiary lipid oxidation products, secondary lipid oxidation products, namely aldehydes with carbon chain length six or seven, are also very reactive with primary amine group (Zamora et al., 2007). If the carbonyl–amine reaction takes place between tertiary lipid oxidation products with free amine group present in PE, the pyrroles produced is likely to be hydrophobic, but if the reaction takes place with amino group of amino acids or protein, the pyrroles produced may be more hydrophilic as shown by mechanism B and C (Hidalgo et al., 2007) in Fig. 3. Two types of pyrroles can be produced during the pyrrolisation process, namely N-substituted pyrroles which are stable and 2-(1-hydroxyalkyl)pyrroles, which are unstable. 2-(1-Hydroxyalkyl)pyrroles can further polymerise to form pyrroles in dimer or polymer form with different antioxidative properties as reported by Hidalgo, Nogales, and Zamora (2003).

The amino group of PE undergoes pyrrolisation 10 times more readily than the amino group of amino acids. This was hypothesised to be due to the close proximity of the generation place of lipid oxidation products to the amino group of PE (Zamora et al., 2005). In emulsions, PE will mainly be present at the oil–water interface. Likewise, tertiary lipid oxidation products, which are more polar than their parent fatty acid will also be located near the oil–water interface, and thereby the reaction between PE and tertiary oxidation products is more likely to occur than the reaction

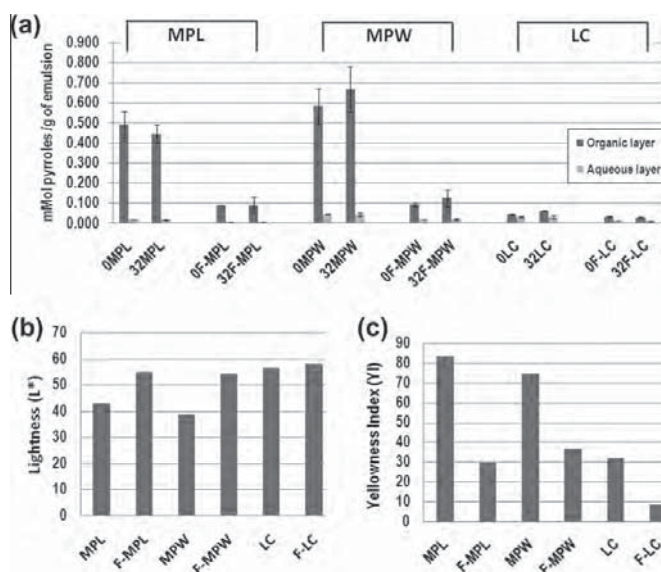


Fig. 2. (a) Comparison of pyrrole content between 0 and 32 days, (b) lightness, (c) yellowness index (YI) of fresh marine PL emulsions on day 0. Values are mean ($n = 2$, SD < 5%).

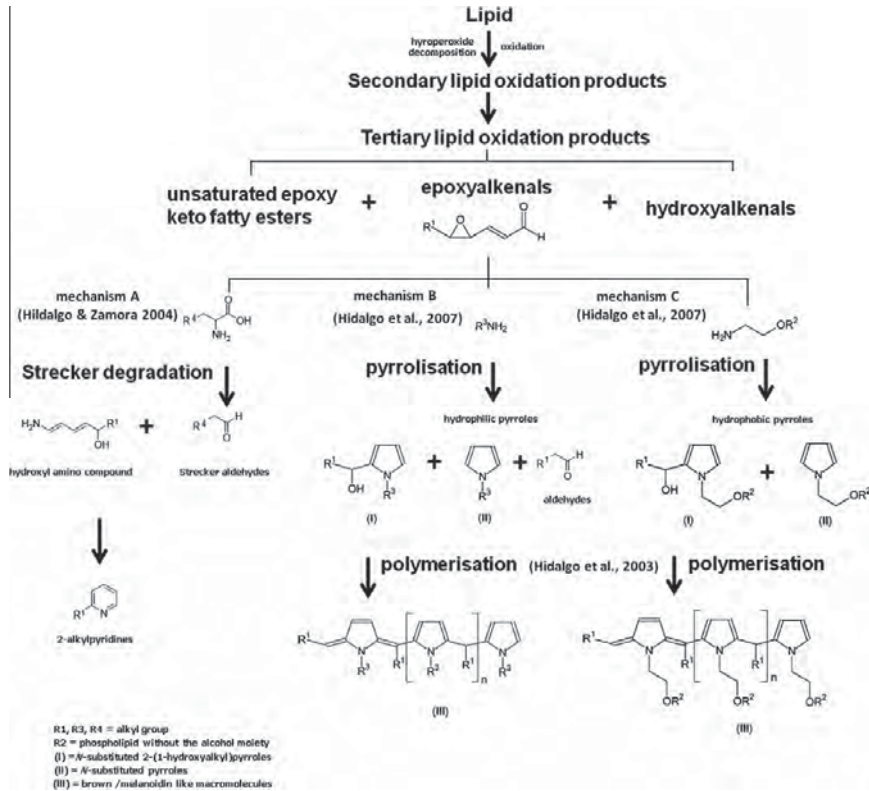


Fig. 3. Proposed mechanisms for non-enzymatic browning development in marine PL.

between tertiary oxidation products and free amino acids as they mainly can be expected to be located in the water phase. In order to study if there is a significant loss of PE in marine PL emulsions during storage, which could indicate pyrrolisation, the determination of PE content through e.g. ^{31}P NMR would be valuable in future studies.

According to Hidalgo et al. (2003), slightly oxidised PE produces pyrroles in a dimer form, which have better antioxidative properties than pyrroles in the polymer form. Further increase of PE oxidation decreased the antioxidative properties of the PE produced pyrroles as most of the dimers were gradually polymerised to form polymers. During the non-enzymatic browning development, two types of reactions are competing with each other: the decrease in antioxidative activity of PE as a consequence of oxidation of the fatty acid and the increase in antioxidative activity of PE as a consequence of carbonyl–amine reactions. The fact that LC had the lowest pyrrole content (either hydrophobic or hydrophilic pyrroles) might indicate that the least pyrrolisation occurred in LC during their manufacturing process. In addition, physical appearance of LC with a light brown colour might also indicate that most of the pyrroles in LC were present in their dimer form and thus gave better protection against oxidation (Hidalgo et al., 2003). Moreover, even though there was a high pyrrole content in MPW and MPL raw materials, these pyrroles did not seem to protect the marine PLE against oxidation. This may indicate that the pyrroles present in these two raw materials were primarily in the polymer form.

3.3.3. Colour changes

Pyrroles from non-enzymatic browning are responsible for brown colour development (Zamora et al., 2005). To study the colour differences due to the non-enzymatic browning reactions, lightness (L^*) and yellow index (YI) were measured in marine PL emulsions during storage. No significant ($p > 0.05$) colour changes were found in marine PL emulsions during 32 days of storage at 2°C (and therefore only data from day 0 are shown in Fig. 2b and c). Due to the high initial content of pyrrole in marine PL materials, the colour changes of marine PL emulsions upon storage might be difficult to observe. However, colour differences between the different formulations of marine PL emulsions could easily be observed (Fig. 2b and c). LC emulsions had higher lightness and lower YI than both MPW and MPL emulsions. This finding might be due to the lower pyrrole content in raw material LC and higher pyrrole content in MPW and MPL as shown in Fig. 2a. Comparison of MPW and MPL emulsions showed that MPL emulsions were lighter and had a lower YI when compared to MPW emulsion. This might be due to the lower pyrrole content in MPL as a result of the ability of ethoxyquin to protect the lipids against oxidation.

4. Conclusion

The oxidative stability of marine PL emulsions was significantly influenced by the chemical composition of marine PL used for emulsions preparation. The stability of the emulsions varied in

relation to the composition of the marine PL preparations, the purity and the type and content of antioxidants and lipids as well as the presence of pyrrolisation compounds and Strecker aldehydes. Emulsions with good oxidative stability could be prepared from marine PL with higher purity (lower initial hydroperoxides and iron content) and higher content of PL and antioxidants (tocopherol or ethoxyquin), and lower TAG content. The effect on lipid oxidation of replacing some of the PL with fish oil was not straightforward. For LC emulsions, fish oil addition decreased oxidative stability, whereas the opposite was observed for MPW and MPL emulsions. These differences were partly due different levels of tocopherol and PL in the raw materials. Non-enzymatic browning reactions were suggested to occur in marine PL mainly during their manufacturing processes. There was a minor increase in SD products and no PL pyrrolisation in the marine PL emulsion during storage at 2 °C. In addition, the SD reaction in marine PL emulsion appeared to be dependent on the level of the amino acids residues present in marine PL. No clear conclusion could be made about the effect of non-enzymatic browning reactions on lipid oxidation and further investigations are required to elucidate this matter.

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References

- AOCS Official method Ce 2-66 (1998). *Preparation of methyl esters of long chain fatty acids*. Champaign, IL: AOCS.
- AOCS Official method Ce 1b-89 (1998). *Fatty acids composition of marine oils by GLC*. Champaign, IL: AOCS.
- AOCS Official method Ce 8-89 (1998). *Determination of tocopherols and tocotrienols in vegetable oils and fats by HPLC*. Champaign, IL: AOCS.
- AOCS Official method Ce 5a-40 (1998). *Free fatty acids*. Champaign, IL: AOCS.
- Applegate, K. R., & Glomset, J. A. (1986). Computer-based modeling of the conformation and packing properties of docosahexaenoic acid. *Journal of Lipid Research*, 27, 658–680.
- Boyd, L. C., Nwosu, V. C., Young, C. L., & MacMillian, L. (1998). Monitoring lipid oxidation and antioxidant effects of phospholipids by headspace gas chromatographic analyses of rancimat trapped volatiles. *Journal of Food Lipids*, 5, 269–282.
- Chung, H. Y., Yung, I. K. S., & Kim, J. S. (2001). Comparison of volatile components in dried scallops (*Chlamys farreri* and *Patinopecten yessoensis*) prepared by boiling and steaming methods. *Journal of Agricultural and Food Chemistry*, 49, 192–202.
- Francis, F. J., & Clydesdale, F. H. (1975). *Food colourimetry: Theory and application*. Westport, CT: AVI Publishing.
- Henna Lu, F. S., Nielsen, N. S., Timm-Heinrich, M., & Jacobsen, C. (2011). Oxidative stability of marine phospholipids in the liposomal form and their applications. *Lipids*, 46, 3–23.
- Hidalgo, F., Alaiiz, M., & Zamora, R. (1999). Effect of pH and temperature on comparative non-enzymatic browning of proteins produced by oxidized lipids and carbohydrate. *Journal of Agricultural and Food Chemistry*, 47, 742–747.
- Hidalgo, F. J., Mercedes Leaoan, M., Nogales, F., & Zamora, R. (2007). Effect of tocopherols in the antioxidative activity of oxidized lipid-amine reaction products. *Journal of Agricultural and Food Chemistry*, 55, 4436–4442.
- Hidalgo, F. J., Nogales, F., & Zamora, R. (2003). Effect of the pyrrole polymerization mechanism on the antioxidative activity of nonenzymatic browning reactions. *Journal of Agricultural and Food Chemistry*, 51, 5703–5708.
- Hidalgo, F. J., Nogales, F., & Zamora, R. (2005). Changes produced in the antioxidative activity of phospholipids as a consequence of their oxidation. *Journal of Agricultural and Food Chemistry*, 53, 659–662.
- Hidalgo, F. J., & Zamora, R. (2004). Strecker-type degradation produced by the lipid oxidation products 4,5-epoxy-2-alkenals. *Journal of Agriculture and Food Chemistry*, 52, 7126–7131.
- Ierna, M., Kerr, A., Scales, H., Berge, K., & Griinari, M. (2010). Supplementation of diet with krill oil protects against experimental rheumatoid arthritis. *BMC Musculoskeletal Disorders*, 11, 136.
- International IDF Standard 74 A (1991). *Milk and milk products: Determination of the iron content*. Brussels: International Dairy Federation.
- Iverson, J. S., Lang, L. C. S., & Cooper, M. H. (2001). Comparison of the bligh and dyer and folch methods for total lipid determination in a broad range of marine tissue. *Lipids*, 36, 1283–1287.
- Jacobsen, C. (2008). Antioxidant strategies for preventing oxidative flavour deterioration of foods enriched with n-3 polyunsaturated lipids: A comparative evaluation. *Trends in Food Science & Technology*, 19, 76–93.
- Linder, M., & Ackman, R. G. (2002). Volatile compounds recovered by solid phase microextraction from fresh adductor muscle and total lipids of sea scallop (*Placopecten magellanicus*) from Georges Bank (Nova Scotia). *Journal of Food Chemistry*, 67, 2032–2037.
- Lu, F. S. H., Nielsen, N. S., Baron, C., & Jacobsen, C. (in press). Physico-chemical properties of marine phospholipid emulsions. *Journal of the American Oil Chemists' Society*.
- Lu, F. S. H., Nielsen, N. S., Jacobsen, C. (submitted for publication). Short Communication: Comparison of two methods for extraction of volatiles from marine phospholipids emulsions. *European Journal of Lipid Science and Technology*.
- Moriya, H., Kuniminato, T., Hosokawa, M., Fukunaga, K., Nishiyama, T., & Miyashita, K. (2007). Oxidative stability of salmon and herring roe lipids and their dietary effect on plasma cholesterol levels of rats. *Fisheries Science*, 73, 668–674.
- Mozuraityte, R., Rustad, T. T., & Storro, I. (2008). The role of iron in peroxidation of polyunsaturated fatty acids in liposomes. *Journal of Agricultural and Food Chemistry*, 56, 537–543.
- Peng, J. L., Larondelle, Y., Pham, D., Ackman, R. G., & Rollin, X. (2003). Polyunsaturated fatty acid profiles of whole body phospholipids and triacylglycerols in anadromous and landlocked Atlantic salmon (*Salmo salar* L.) fry. *Comparative Biochemistry and Physiology B*, 134, 335–348.
- 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.
- Timm-Heinrich, M., Xuebing, X., Nielsen, N. S., & Jacobsen, C. (2003). Oxidative stability of milk drinks containing structured lipids produced from sunflower oil and caprylic acid. *European Journal of Lipid Science and Technology*, 105, 459–470.
- Ventanas, S., Estevez, M., & Delgado, C. L. (2007). Phospholipid oxidation, non-enzymatic browning development and volatile compounds generation in model systems containing liposomes from porcine *Longissimus dorsi* and selected amino acids. *European Food Research and Technology*, 225, 665–675.
- Wijendran, V., Huang, M. C., Diao, G. Y., Boehm, G., Nathanielsz, P. W., & Brenna, J. T. (2002). Efficacy of dietary arachidonic acid provided as triglyceride or phospholipid as substrates for brain arachidonic acid accretion in baboon neonates. *Pediatric Research*, 51, 265–272.
- Zamora, R., Gallardo, E., & Hidalgo, F. (2007). Strecker degradation of phenylalanine initiated by 2,4-decadienal or methyl 13-oxooctadeca-9, 11-dienoate in model systems. *Journal of Agricultural and Food Chemistry*, 55, 1308–1314.
- Zamora, R., Nogales, F., & Hidalgo, F. J. (2005). Phospholipid oxidation and nonenzymatic browning development in phosphatidylethanolamine/ribose/lysine model systems. *European Food Research and Technology*, 220, 459–465.
- Zamora, R., Rios, J. J., & Hidalgo, F. J. (1994). Formation of volatile pyrrole products from epoxyalkenals/protein reactions. *Journal of Agricultural and Food Chemistry*, 66, 543–546.

PAPER IV

Lu, F. S. H., Nielsen, N, S., Baron, C. P., Diehl, B. W. K., & Jacobsen, C.

Oxidative stability of dispersions prepared from purified marine phospholipid and the role of α -tocopherol.

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Oxidative Stability of Dispersions Prepared from Purified Marine Phospholipid and the Role of α -Tocopherol

F. S. Henna Lu,[†] Nina S. Nielsen,[†] Caroline P. Baron,[†] Bernd W. K. Diehl,[§] and Charlotte Jacobsen^{*,†}

[†]Division of Industrial Food Research, Lipid and Oxidation Group, National Food Institute, Technical University of Denmark, Soltofts Plads, Building 221, 2800 Kgs. Lyngby, Denmark

[§]Spectral Service AG, Emil-Hoffmann-Straße 33, D-50996 Köln, Germany

ABSTRACT: The objective of this study was to investigate the oxidative stability of dispersions prepared from different levels of purified marine phospholipid (PL) obtained by acetone precipitation, with particular focus on the interaction between α -tocopherol and PL in dispersions. This also included the investigation of nonenzymatic browning in purified marine PL dispersions. Dispersions were prepared by high-pressure homogenizer. The oxidative and hydrolytic stabilities of dispersions were investigated by determination of hydroperoxides, secondary volatile oxidation products, and free fatty acids, respectively, during 32 days of storage at 2 °C. Nonenzymatic browning was investigated through measurement of Strecker aldehydes, color changes, and pyrrole content. Dispersions containing α -tocopherol or higher levels of purified marine PL showed a lower increment of volatiles after 32 days storage. The results suggested that tocopherol is an efficient antioxidant in PL dispersions or that the presence of α -tocopherol and pyrroles may be the main reason for the high oxidative stability of purified marine PL dispersions.

KEYWORDS: marine phospholipids, fish oil, oxidative stability, nonenzymatic browning, pyrrolization, Strecker degradation, α -tocopherol

INTRODUCTION

Many studies have shown that marine phospholipids have better oxidative stability than marine triglyceride (TAG) available from fish oil,^{1,2} and most of these studies were carried out on marine phospholipids in liposomal form.^{3–6} The issue of oxidative stability of marine phospholipid (PL) has been reviewed extensively in our previous publication,⁷ and it can be summarized as follows: A high oxidative stability of marine PL might be due to (a) their tight intermolecular packing conformation with the polyunsaturated fatty acids (PUFA) at the sn-2 position of PL^{1,8} and (b) a synergistic effect of phospholipids on the antioxidant activity of α -tocopherol.^{6,9} In addition, recent studies¹⁰ showed that pyrroles formed from nonenzymatic browning reactions between oxidized amino phospholipids/amino acids and fatty acid oxidation products in slightly oxidized marine PL have protective effects against oxidation. Among these factors, a synergistic effect of PL on the antioxidant activity of α -tocopherol seems to be the main reason for the stability of marine PL as suggested by several studies.^{6,9} Furthermore, the antioxidative activity of pyrroles may be greatly increased by the addition of α -tocopherols as a result of synergism between α -tocopherol and pyrroles.¹¹

The mechanism responsible for the synergy of tocopherols and PL is not well understood, but postulated mechanisms are suggested by several studies.^{12,13} Bandarra and co-workers¹² investigated the prevention of oxidation in a refined sardine oil system with added α -tocopherol at 0.04% or with added phosphatidylcholine (PC), phosphatidylethanolamine (PE), and cardiolipin (CL) at 0.5%, respectively. They reported that PC was the most effective individual antioxidant when it was compared to PE, CL, and α -tocopherol, whereas the highest synergistic effect was provided by PE. This phenom-

enon could be due to the easier hydrogen transfer from the amine group of PE to tocopheroxyl radical and regeneration of tocopherol or the secondary antioxidant action of PE in reducing quinines formed during oxidation of tocopherols as suggested by Weng and Gordon.¹³

Our previous study¹⁴ reported that the oxidative stability of marine PL emulsions varied in relation to the chemical composition and purity of marine PL used for their preparation. For instance, marine PLs with high purity (low initial hydroperoxides and iron content), high content of PL and antioxidant (tocopherol or ethoxyquin), and low content of TAG were shown to have high oxidative stability. Moreover, the oxidative stability of marine PL may be influenced by the presence of residues of amino acids, protein, reducing sugar, and also their degradation products such as pyrroles formed via pyrrolization and Strecker aldehydes formed via Strecker degradation (SD).¹⁴ The primary objective of this study was therefore to investigate the oxidative stability of dispersions prepared from purified marine PL in different concentrations. Marine PL was purified by acetone precipitation to eliminate the effect of other factors on lipid oxidation such as content of TAG, antioxidant, or other residues that might be present in marine PL. To the best of our knowledge, the oxidative stability and nonenzymatic browning in dispersions prepared from purified marine PL have not previously been studied. Furthermore, the oxidative stability of purified marine PL was studied in dispersions instead of bulk lipid due to the increasing

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interest in using marine PL dispersion as an n-3 PUFA delivery system. The secondary objective of this study was to investigate the interactions between PL and α -tocopherol in a complex marine PL dispersion system containing nonenzymatic reaction products to obtain a more comprehensive understanding of this interaction.

MATERIALS AND METHODS

Materials. Marine phospholipid (MPW), marine PL extracted from sprat fish meal, was obtained from Triple Nine Pharma (Esbjerg, Denmark). The chemicals sodium acetate and imidazole were obtained from Fluka (Sigma-Aldrich Chemie GmbH, Buchs, Spain) and Merck (Darmstadt, Germany), respectively. Other solvents were of HPLC grade (Lab-Scan, Dublin, Ireland).

Methods. Purification of Marine PL by Acetone Precipitation. Marine PL (MPW) was further isolated from neutral lipids by using an acetone precipitation method as described by Mozuraityte and co-workers⁵ and Schneider and Lovaas¹⁵ with a few modifications. According to Schneider and Lovaas,¹⁵ this method could produce PL with very low lipophilic contamination levels (polychlorinated biphenyls and dioxins), and thus the final products can be used without further purification. A total weight of 130 g marine PL was dissolved in approximately 200 mL chloroform. This solution was then poured into 1000 mL of acetone (approximate ratio of 1:7.7) under vigorous stirring at ambient temperature. The ratio of lipids to solvent was according to Schneider and Lovaas.¹⁵ The mixed solution was kept at -18°C overnight to allow phospholipid precipitation. The acetone was decanted, the precipitates were redissolved in chloroform, and the isolation procedure was repeated once more. The final precipitates (purified PL) were dried under nitrogen for 1 h. The residues of acetone and chloroform were further removed under vacuum at 40°C . To ensure that the production method did not change the fatty acid composition of PL or lipid classes, the fatty acid composition of the final product was checked by GC-FID and the lipid classes were determined again through thin-layer chromatography by TLC-FID Iatroskan MK-V (Iatron Laboratories, Inc., Tokyo, Japan) equipped with Chromo Star v3.24S software (Bruker-Franzen & SCAP, Germany).

Preparation of Marine PL Dispersions. Five different formulations of marine PL dispersions (300 mL for each formulation) were prepared with different levels of purified marine PL (as shown in Table 1). Due to the removal of TAG in purified marine PL, the prepared

Table 1. Experimental Design for Marine PL Dispersions

formulation/ dispersion	added tocopherol (mg/g of PL)	phospholipid (%)	total lipid (%)	acetate- imidazole buffer (%)
APT	0.25	2.0	2.0	98
AP1	0.0	2.0	2.0	98
AP2	0.0	4.0	4.0	96
AP3	0.0	6.0	6.0	94
AP4	0.0	8.0	8.0	92

dispersions contain mainly liposomes, which have a particle size of $0.1\ \mu\text{m}$ as also reported in our previous study.¹⁶ One of the formulations (APT) had added α -tocopherol. Dispersions were prepared in two steps; pre-emulsification and homogenization. In the pre-emulsification step, marine PL was added to the buffer over 1 min under vigorous mixing (19000 rpm) with an Ultra-Turrax (Ystral, Ballrechten-Dottingen, Germany) followed by 2 min of mixing at the same speed. All pre-dispersions were subsequently homogenized in a Panda high-pressure table homogenizer (GEA Niro Soavi SPA, Parma, Italy) using pressures of 800 and 80 bar for the first and second stages, respectively. After homogenization, 1 mL of sodium azide (10%) was added to each sample (220 g) to inhibit microbial growth. Dispersions were stored in closed 250 mL blue-cap bottles at 2°C in

darkness. The blue-cap bottles were opened for sampling on 0, 4, 8, 16, and 32 days of storage; that is, samples were taken from the same bottle. Samples were flushed with nitrogen and stored at -40°C until further analysis. Samples were analyzed for their hydrolytic stability, which included the measurement of free fatty acids (FFA) and PL composition by ^{31}P NMR. In terms of oxidative stability, samples were analyzed for tocopherol content, peroxide value (PV), and secondary volatile oxidation products through solid-phase microextraction (SPME) GC-MS at five time intervals as mentioned earlier. In addition, SD was studied by measurement of 3-methylbutanol content through SPME-GC-MS. To study the PL pyrrrolization in marine PL dispersions, the content of pyrroles and color changes of marine PL dispersions were determined before and after 32 days of storage.

Characterization of Marine PL. (a) Determination of Tocopherol Content. Approximately 0.5 g of marine PL was used for extraction with heptane (5 mL), and the extract was analyzed for tocopherol content by HPLC analysis (Agilent 1100 series, Agilent Technologies, Palo Alto, CA, USA). For determination of tocopherol, a Water Spherisorb (R) silica column ($4.6 \times 150\ \text{mm}$, i.d. = $3\ \mu\text{m}$; Waters Corp., Milford, MA, USA) was used. The mobile phase consisted of heptane and isopropanol (100:0.4, respectively) and was introduced at a flow rate of 1 mL/min. Tocopherols were detected with a fluorescence detector (FLD) at 290 nm as excitation wavelength and at 330 nm as emission wavelength according to AOCs Official Method Ce 8-89.¹⁷ The analysis was performed in duplicate.

(b) Determination of Fatty Acid Profile of the Different Lipid Classes and PL Profile. The different lipid classes of marine PL were measured by TLC-FID Iatroskan MK-V (Iatron Laboratories, Inc., Tokyo, Japan) with Chromo Star v3.24S software (Bruker-Franzen & SCAP, Germany). The 10 silica gel chromorods SIII (Iatron Laboratories Inc.) were blank scanned twice immediately before sample application to remove impurities. Lipids (15 mg/mL chloroform/methanol, 2:1) were then spotted on the chromorods using a semiautomatic sample spotter (SES GmbH – Analyse system, Germany). The separation of lipid classes was done by development in *n*-heptane/diethyl ether/formic acid (70:10:0.02, v/v/v). The neutral lipids (NL) consisting of triglyceride (TAG), free fatty acids (FFA), and cholesterol (CHO) were separated from polar lipids and non-lipid material. After development, the rods were dried in an oven at 120°C for 2 min and then fully scanned in the Iatroskan MK-V. The air and hydrogen flow rates were set at 200 L/min and 160 mL/min, respectively. The scan speed was set at 30 s/rod. The lipid class of marine PL was expressed as the mean percentage of three analyses from each sample. For fatty acid composition, approximately 0.5 mL of marine phospholipids in chloroform (with a concentration of 10–20 mg/mL) was transferred to a Sep-Pak column containing 500 mg of aminopropyl-modified silica (Waters Corp.) for lipid separation. A mixture of $2 \times 2\ \text{mL}$ of chloroform and 2-propanol (ratio 2:1) was used to elute the neutral lipid fraction (NL), whereas $3 \times 2\ \text{mL}$ of methanol was used to elute the PL fraction by gravity. Eluates were evaporated under nitrogen and methylated according to AOCs Official Method Ce 2-66,¹⁸ followed by separation through gas chromatography (HP 5890 A, Hewlett Packard, Palo Alto, CA, USA) with an Omegawax 320 column (Supelco, PA, USA) according to the method described by AOCs Official Method Ce 1b-89.¹⁹ The fatty acid composition was determined in duplicate. The PL profile of marine PL was determined through ^{31}P NMR by Spectra Service GmbH (Cologne, Germany). All spectra were acquired using an Avance III 600 NMR spectrometer (Bruker, Karlsruhe, Germany) and a magnetic flux density = 14.1 Tesla QNP cryo probe head equipped with automated sample changer Bruker B-ACS 120. Computer Intel Core2 Duo 2.4 GHz with MS Windows XP and Bruker TopSpin 2.1 was used for acquisition, and Bruker TopSpin 2.1 was used for processing.

(c) Determination of Iron Content. Marine PL was digested with 5 mL of HNO_3 (65%) and 150 μL of HCl (37%) in a microwave oven at 1400 W (Anto Paar multiwave 3000, Graz, Austria) for 1 h. The samples were further digested with 150 μL of H_2O_2 for another 45 min. Thereafter, the iron concentration was measured by an atomic absorption spectrophotometer (AAS 3300, Perkin Elmer, Boston, MA,

USA). Two digestions were made from each sample, and the measurement was performed in duplicate.

(d) **Determination of Peroxide Value and Free Fatty Acid Content.** PV was measured on marine PL by the colorimetric ferric-thiocyanate method at 500 nm using a spectrophotometer (Shimadzu UV-160A, UV-Vis, Shimadzu Corp., Kyoto, Japan) as described by IDF²⁰ and Shantha and Decker.²¹ FFA values of marine PL were determined according to AOCS Official Method Ce 5a-40,²² and the measurement was performed in duplicate.

(e) **Determination of Induction Period by Accelerated Oxidation Stability Measurement.** The induction periods of both untreated marine PL (MPW) and purified marine PL (AP) were measured electronically at 60 °C under oxygen pressure (5 bar) in an Oxipres apparatus (Mikrolab Aarhus A/S, Højbjerg, Denmark). Samples (5 g) were weighed into reaction flasks (100 mL), and the drop in the oxygen pressure in the reaction flasks as a result of oxygen consumption was recorded by using a picolog recorder. The induction period was determined in duplicate as the crossing point of the tangents to the curve.

Determination of Lipid Oxidation and Nonenzymatic Browning in Dispersions. (a) **Determination of Peroxide Value, Free Fatty Acids, and Tocopherol Content.** Lipids were extracted from the dispersions according to the Bligh and Dyer method using a reduced amount of the chloroform/methanol (1:1 w/w) solvent.²³ Two extractions were made from each sample, and the measurement was performed in duplicate. Both PV and FFA were measured according to the methods mentioned earlier. For tocopherol determination, lipid extracts (1–2 g) from Bligh and Dyer were weighed and evaporated under nitrogen prior to analysis by using the same method as mentioned earlier.

(b) **Headspace Analysis Using Solid-Phase Microextraction (SPME) GC-MS.** Approximately 1 g of the sample, together with 30 mg of internal standard (10 µg/g of 4-methyl-1-pentanol in rapeseed oil), was mixed on a whirly mixer for 30 s in a 10 mL vial. The sample was equilibrated for 3 min at a temperature of 60 °C, followed by extraction for 45 min at the same temperature while the sample was agitated at 500 rpm. Extraction of headspace volatiles was done by using a 50/30 µm CAR/PDMS SPME fiber (Supelco) installed on a CTC Combi Pal (CTC Analytics, Waldbronn, Germany). Volatiles were desorbed in the injection port of the gas chromatograph (HP 6890 Series, Hewlett Packard, Palo Alto, CA, USA; column, DB-1701, 30 m × 0.25 mm × 1.0 µm; J&W Scientific, Folsom, CA, USA) for 60 s at 220 °C. The oven program had an initial temperature of 35 °C for 3 min, with increment of 3.0 °C/min to 140 °C, then increment of 5.0 °C/min to 170 °C, and increment of 10.0 °C/min to 240 °C, at which the temperature was held for 8 min. The individual compounds were analyzed by mass spectrometry (HP 5973 inert mass-selective detector, Agilent Technologies, USA; electron ionization mode, 70 eV; mass to charge ratio scan between 30 and 250). To investigate SD in purified PL dispersions, 3-methylbutanal was selected for quantification, whereas for lipid oxidation investigation, six n-3 derived secondary volatiles were selected for quantification: (E,Z)-2,4-heptadienal, (E,E)-2,4-heptadienal, (E)-2-pentenal, (E,Z)-2,6-nonadienal, (Z)-4-heptenal, and 2-ethylfuran.

Calibration curves were made by dissolving the related volatile standards in rapeseed oil followed by dilution to obtain different concentrations (0.1–100 µg/g). In this study calibration curves were parallel shifted to obtain positive values. The given values (in ng/g) of the volatiles are thus estimated values and should therefore not be used for comparison to other studies. Measurements were made in triplicates on each sample.

(c) **Determination of Phospholipids Composition by ³¹P NMR.** PL composition of purified marine PL dispersions was determined through ³¹P NMR by Spectra Service GmbH (Cologne, Germany) using the same method as used for neat MPW. However, only a single measurement was made for this analysis.

(d) **Determination of Pyrrole Content and Color Changes.** Dispersion prepared from purified marine PL (3 mL) was extracted twice with 6 mL of chloroform/methanol (2:1), and the resulting organic extracts (chloroform phase) were analyzed for pyrrole content

and color changes. Organic extract (0.5 g) was dried under nitrogen, and 1 mL of 150 mM sodium phosphate (pH 7) containing 3% sodium dodecyl sulfate (SDS) was added. This solution was then treated with Ehrlich reagent (700 µL of reagent A and 170 µL of reagent B). Reagent A was prepared by mixing 2 mL of ethanol with 8 mL of HCl (2.5 N), whereas reagent B was prepared by suspending 200 mg of *p*-(dimethylamino)benzaldehyde in 10 mL of reagent A. The final solution was incubated at 45 °C for 30 min. The absorbance of the maximum at 570 nm was measured against a blank prepared under the same conditions but without *p*-(dimethylamino)benzaldehyde. Two extractions were made from each sample, and the measurement was performed in duplicate. Pyrrole content was quantified by an authentic external standard, 1-(4-methoxyphenyl)-1H-pyrrole (this standard gives absorbance at 570 nm). The pyrrole concentration is thus given as millimoles of 1-(4-methoxyphenyl)-1H-pyrrole per gram of dispersion. Color changes were measured on the organic extract as well using a spectrophotometer (X-Rite, Inc., Grandville, MI, USA). The instrument was calibrated before each measurement, and the results were recorded using the CIE color system profile of *L** (lightness), *a** (redness/greenness), and *b** (yellowness/blueness). In addition, a yellowness index (YI) was calculated according to the method of Francis and Clydesdale:²⁴ YI = 142.86*b**/*L**. Two extractions were performed on each sample, and the measurement was performed in duplicate.

Statistical Analysis. The obtained data, PV, FFA, color, pyrrole, and volatile measurements were subjected to one-way ANOVA, and comparison among samples was performed with Tukey's multiple-comparison test using a statistical package program Minitab 16 (Minitab Inc., State College, PA, USA). Significant differences were accepted at *P* < 0.05.

RESULTS AND DISCUSSION

Chemical Composition of Purified Marine PL. In this study, marine PL (MPW) was purified through acetone precipitation with the purpose to remove TAGs and also other nonpolar lipids and thus to increase the percentage of PL in marine PL. The PL percentage increased from 41.50 to 66.23%, whereas all TAGs were removed from MPW after acetone precipitation (Table 2). In general, purified marine PL had higher contents of PC, PE, and phosphatidylinositol (PI) than untreated marine PL, with increments of 3.04, 4.51, and 0.66% (absolute values), respectively. However, purified marine PL also had a higher level of lysoPL, approximately 11% (Table 2), indicating hydrolysis of PL during acetone precipitation. Surprisingly, the content of FFA in purified marine PL did not increase as expected but slightly decreased after the acetone treatment. This finding suggested that part of the FFA was removed by acetone treatment. In addition to hydrolysis, purified marine PL had a higher degree of oxidation than untreated marine PL. This could be observed by the higher PV and initial n-3 derived volatiles in AP as compared to MPW. The decrease in the oxidative stability of AP might be related to the removal of the lipid-soluble antioxidant α -tocopherol during the purification process. In terms of the fatty acid composition of MPW, the PL fraction contained higher levels of EPA and DHA as compared to the NL fraction (Table 3). Thus, the total EPA and DHA content in the PL fraction was approximately 45% as compared to 20% in the NL fraction. This composition was in agreement with the results from other studies.²⁵ In general, the fatty acid composition of the PL fraction of MPW was different from the fatty acid composition of AP. The main differences between these two marine PLs were (a) the lower content of EPA and DHA, which was most likely due to the oxidation during acetone precipitation, and (b) the higher content of other unidentified fatty acids in AP as compared to that of MPW.

Table 2. Composition of Marine PL before and after Acetone Precipitation

name	MPW	AP
	sprat fish meal	MPW after acetone precipitation
total phospholipids (%)	41.50	66.23
phosphatidylcholine, PC (%)	18.30	21.34
phosphatidylethanolamine, PE (%)	4.70	9.21
phosphatidylinositol, PI (%)	2.10	2.76
sphingomyelin, SPM (%)	— ^a	—
lysophosphatidylcholine, LPC (%)	3.40	11.15
other phospholipids	8.90	21.77
triglycerides, TAGs	40.0	—
cholesterol, CHO	2.0	ND ^b
free fatty acids	16.0	11.0
peroxide value (mequiv/kg)	0.81 ± 0.04	1.66 ± 0.21
initial n-3 derived volatiles (mg/kg)	64.2	75.6
Strecker volatiles		
3-methylbutanal (mg/kg)	0.36 ± 0.07	0.12 ± 0.03
α-tocopherol (mg/kg)	73.4	
induction period, IP (min)	1569 ± 23	41 ± 6
after addition of α-tocopherol (600 mg/kg)		IP was not attained even after 6 days of incubation

^a—, not detectable. ^bND, not determined.

Hydrolytic Stability of Purified Marine PL Dispersions.

Acetone precipitation increased PL hydrolysis in AP preparation as shown by its higher level of LPC content (Table 1), but the resulting dispersions prepared from this marine PL did not hydrolyze further and showed the same degree of hydrolysis after storage. With regard to the phospholipid hydrolysis during the acetone precipitation, the phospholipid hydrolysis was most likely initiated by the residues of water (approximately 0.2–0.5 %) or other impurities present in acetone used for precipitation.²⁶ Another possibility is that the solubilization of marine phospholipids in acetone solution increased the phospholipid hydrolysis and its catalysis by H ions stemming from the free fatty acids. As shown in Table 4, there were no significant differences ($P > 0.05$) in PC, LPC, PE, and LPE before and after 32 days of storage at 2 °C. The same observation was obtained for free fatty acid measurement in the dispersions (data not shown). This might be due to the neutral-pH imidazole buffer used for dispersion preparation. According to Gritt and co-workers,²⁷ hydrolysis of PL will be minimal at neutral pH as PL hydrolysis is catalyzed by hydroxyl and hydrogen ions.

Oxidative Stability of Purified Marine PL Dispersions.

All dispersions prepared from purified marine phospholipids were found to contain particles, which have a size of approximately 0.1 μm that might indicate the presence of liposomes,¹⁶ and particles that have a size of approximately 100 μm (data not shown). Because all of the dispersions were found to have the same particle size distribution, the effect of the particle size toward oxidative stability of dispersion will not

Table 3. Fatty Acid Compositions of Marine PL before and after Acetone Precipitation^a

fatty acid	MPW (before)		AP (after)
	neutral lipids fraction NL (%)	phospholipids PL (%)	total lipids, PL (%)
C14:0	5.71	1.72	1.45
C16:0	17.51	27.32	23.67
C16:1 (n-7)	6.25	1.74	0.24
C16:2 (n-4)	0.29	0.41	0.69
C18:0	2.67	2.46	4.76
C18:1 (n-9)	17.21	14.06	13.40
C18:1 (n-7)	0.30	0.11	0.05
C18:2 (n-6)	2.09	1.02	1.45
C18:3 (n-6)	1.86	0.68	0.07
C18:3 (n-3)	0.00	0.00	0.00
C18:4 (n-3)	3.44	0.64	0.00
C20:1 (n-9)	5.59	0.14	0.13
C20:4 (n-6)	0.49	1.23	1.29
C20:5 (n-3)EPA	7.83	12.53	7.30
C22:1 (n-11)	7.79	0.00	0.13
C22:6 (n-3)DHA	12.63	32.79	27.4
C24:1 (n-9)	1.10	1.87	1.90
others ^b	2.98	0.50	16.07
EPA + DHA	20.45	45.32	34.70
n-3	26.16	46.76	35.85
n-6	4.82	2.93	2.86
n-9	24.36	16.07	15.43
SAFA	26.71	31.5	30.40
MUFA	39.05	17.92	15.89
PUFA	31.27	50.09	39.40
total	100.0	100.0	100.0

^aValues are means ($n = 2$, standard deviation < 5%). ^bUnidentified fatty acids.

Table 4. Comparison of Phospholipid Content in AP Dispersions before and after 32 Days of Storage at 2 °C by ³¹P NMR (Weight Percent)^a

formulation	PC	2LPC	PE	LPE	total PL
0 APT	0.47	0.25	0.17	0.06	1.47
32 APT	0.41	0.22	0.15	0.04	1.27
0 AP1	0.43	0.22	0.14	0.06	1.38
32 AP1	0.40	0.22	0.14	0.04	1.25
0 AP2	0.95	0.47	0.41	0.10	2.96
32 AP2	0.81	0.44	0.27	0.09	2.58
0 AP3	1.26	0.68	0.56	0.14	4.05
32 AP3	1.27	0.66	0.38	0.13	3.85
0 AP4	1.66	0.90	0.73	0.21	5.38
32 AP4	1.66	0.89	0.69	0.17	5.18

^aOnly single measurement was made, $n = 1$ with 5 % detection limit. The data in this table are used for relative comparison and therefore are different from the total lipid percentages in Table 1.

be further discussed. Dispersions containing higher percentages of purified marine PL (AP3 and AP4) showed significantly lower ($P < 0.05$) PV increment during storage than dispersions containing lower percentages of purified marine PL, namely, AP1, APT, and AP2 (Figure 1). PV did not increase in most of

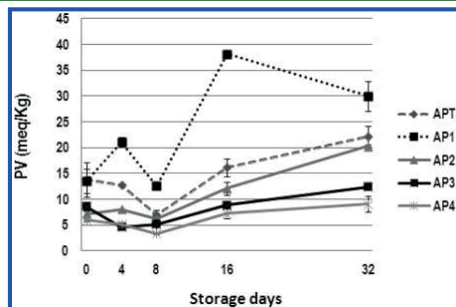


Figure 1. Measurement of PV in AP dispersions during 32 days of storage at 2 °C. Values are the mean \pm standard deviation ($n = 2$).

the dispersions (except AP1) during the first 4 days of storage but slightly decreased on day 8, and it increased again thereafter. AP3 and AP4 seemed to be almost stable with regard to PV development. However, PV measurement was to some extent contradictory to the data obtained from the secondary volatile measurement (Figure 2a,b). For instance, AP4 had the lowest PV during the entire storage period, but had the highest levels of (*Z*)-4-heptenal and (*E*)-2-pentenal after 32 days of storage due to the fast decomposition of hydroperoxides in marine PL.^{14,28} In general, the concentrations of n-3 derived volatiles, namely, (*E*)-2-pentenal, (*E,Z*)-2,6-nonadienal, (*Z*)-4-heptenal, and 2-ethylfuran, increased with increasing percentage of purified marine PL from AP1 to AP4 dispersions except for (*E,E*)-2,4-heptadienal and (*E,Z*)-2,4-heptadienal, which did not show clear differences among the dispersions (data not shown). In addition, the development of volatiles during storage as illustrated by (*Z*)-4-heptenal showed that volatiles slightly increased from day 0 to day 32 (Figure 2a). Interestingly, the increment during storage was lower in dispersions with higher levels of AP (AP3 and AP4) or with tocopherol added (APT). For example, the increment of (*Z*)-4-heptenal (ng/g dispersion or ng/g AP) during storage was as follows: 21 or 1050 in APT, 28 or 1400 in AP1, 30 or 758 in AP2, 23 or 389 in AP3, and 10 or 129 in AP4, respectively. The same trend of increment was obtained for (*E,Z*)-2,6-nonadienal. Hence, the high concentration of volatiles found in AP3 and AP4 at day 32 was not due to the increment of oxidation during storage, but due to the high level of initial volatiles in these dispersions even at day 0. The finding of this study supported the findings of many other studies^{1,2} that dispersions prepared from purified marine PL showed a high oxidative stability, as also illustrated by lower volatile increment in AP3 and AP4 dispersions. Furthermore, the lower volatile increment in APT dispersion containing α -tocopherol as compared to AP1 dispersion despite their same level of PL indicated that tocopherol is an efficient antioxidant in PL dispersions. In contrast to the development behavior of (*Z*)-4-heptenal and (*E,Z*)-2,6-nonadienal, a decreasing trend from 0 to 32 days was observed for (*E*)-2-pentenal, especially in dispersions AP2, AP3, and AP4, whereas this volatile remained

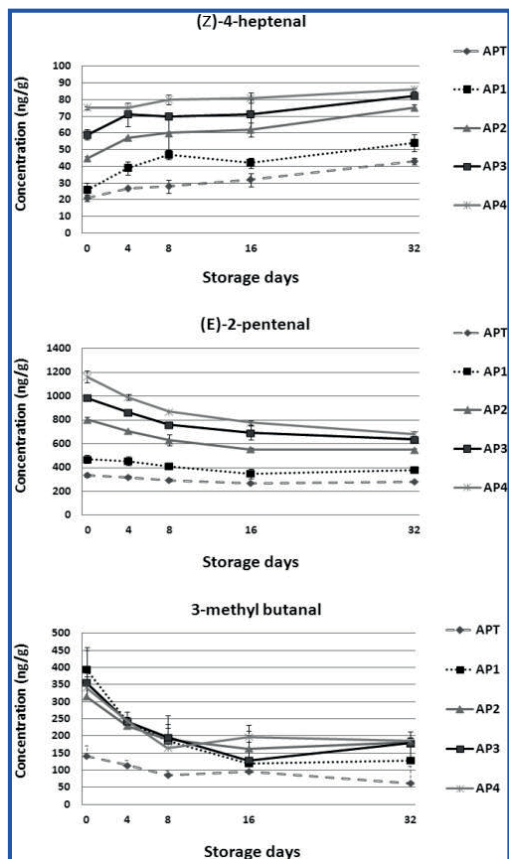


Figure 2. Measurement of (a) (*Z*)-4-heptenal, (b) (*E*)-2-pentenal, and (c) 3-methylbutanal in AP dispersions during 32 days of storage at 2 °C. Values are the mean \pm standard deviation ($n = 2$).

almost constant in AP1 and APT upon 32 days of storage (Figure 2b). The decrement of (*E*)-2-pentenal (ng/g dispersion) during storage was as follows: 55 in APT, 89 in AP1, 254 in AP2, 349 in AP3, and 479 in AP4, respectively. The largest decrement was observed in dispersion containing the highest level of AP. This was also the case for 2-ethylfuran. This phenomenon might be associated with the involvement of these lipid volatiles in nonenzymatic browning, which includes both pyrrolization and SD.

Nonenzymatic Browning in Purified Marine PL Dispersions. Strecker Degradation. In addition to lipid-derived volatiles, secondary volatiles derived from degradation of amino acid residues through SD were found in purified marine PL dispersions. For instance, 3-methylbutanal (Figure 2c) is a Strecker aldehyde derived from the amino acid leucine.^{14,29} As suggested in our previous study,¹⁴ it is speculated that most of the Strecker aldehydes in marine PL are produced mainly during the marine PL manufacturing process, which is carried out at high temperature. Strecker aldehydes are produced from amino acid residues via reaction with tertiary lipid oxidation products such as unsaturated epoxy

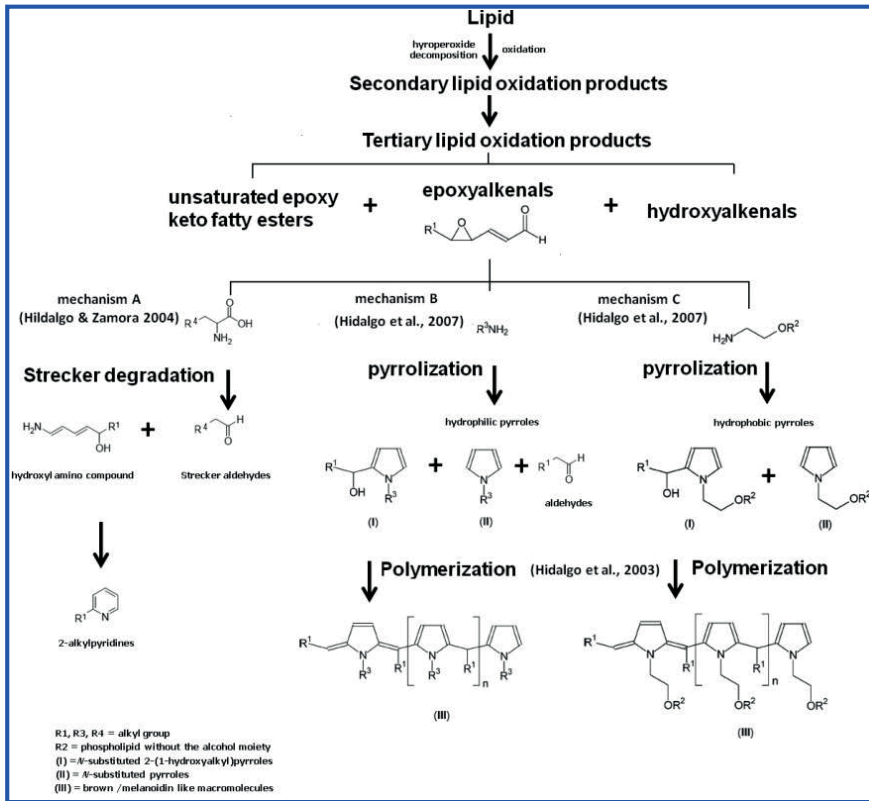


Figure 3. Proposed mechanisms for nonenzymatic browning reactions in marine PL dispersion.

keto fatty esters, epoxyalkenals, and hydroxyalkenals (Figure 3). The presence of two oxygenated function groups in the tertiary lipid oxidation products, namely, one carbonyl group and one epoxy or hydroxyl group, is required for the SD reaction to occur as shown in mechanism A in Figure 3.³⁰ In addition, secondary lipid oxidation products such as alkadienals and ketodienes may also degrade amino acids to their corresponding Strecker aldehydes under appropriate conditions when they undergo further oxidation.³¹

Although the typical SD occurs at high temperature, our previous study¹⁴ reported that SD of amino acids occurred at low rates in marine PL emulsions during 32 days at 2 °C. This finding is in agreement with several other studies, which reported the occurrence of SD of amino acids with α -dicarbonyl or tertiary lipid oxidation products at low temperatures such as 25 °C^{29,32} or 37 °C.³⁰ For instance, Ventanas and co-workers²⁹ reported the occurrence of lipid oxidation, SD, and nonenzymatic browning in a sterile meat model system containing selected amino acids and liposomes after 35 days of incubation at 25 °C under pro-oxidative conditions. As shown in Figure 2, 3-methylbutanal was found in marine PL dispersion on day 0 even before the storage due to its presence in untreated marine PL and, therefore, also in purified marine PL (AP). However, purified marine PL had a much lower concentration of 3-methylbutanal as compared to untreated

marine PL (MPW) as shown in Table 2. Dispersion prepared from purified marine PL did not contain Strecker aldehydes such as dimethyl disulfide, dimethyl trisulfide, pyridines, 2-methylbutanal, and 2-methylpropanal, which were previously reported in MPW.¹⁴ In general, volatiles data showed that all purified marine PL dispersions (AP1–AP4) had the same level of Strecker aldehydes despite their different levels of AP. In other words, AP1 had a higher level of 3-methylbutanal per kilogram of AP as compared to APT, AP2, AP3, and AP4 (19.70 vs 7.1, 7.88, 5.92, and 4.26 mg/kg, respectively). This observation might imply a higher degree of SD in AP1 dispersion, followed by AP2, APT, AP3, and AP4. However, the decrease of 3-methylbutanal over time might be due to the sampling technique that caused the release of volatiles from the storage bottle as it was opened for sampling every time. Further investigation is required to elucidate this matter.

Pyrrrolization and Color Changes. The content of pyrroles might increase in purified marine PL (AP) after acetone treatment due to the increase of its brownness as observed visually. As suggested in our previous study,¹⁴ pyrrolization of tertiary lipid oxidation products with the amine group from PE may form hydrophobic pyrroles, whereas its pyrrolization with amino acids may form hydrophilic pyrroles (mechanisms B and C in Figure 3). In this study, pyrrolization in purified marine PL dispersions was investigated through measurement of hydro-

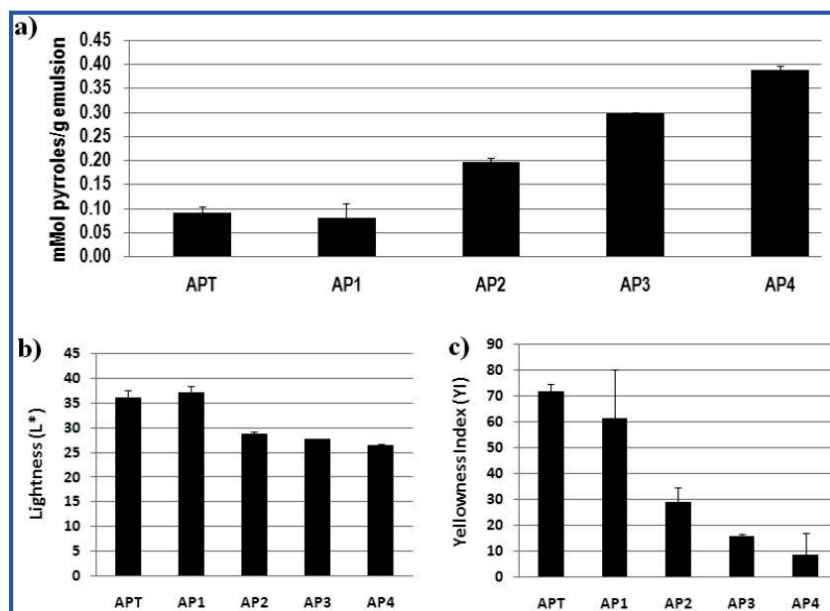


Figure 4. Comparison of (a) pyrrole content (hydrophobic), (b) lightness (L^*), and (c) yellowness index (YI) of marine PL dispersions on day 0. Values are the mean \pm standard deviation ($n = 2$).

phobic pyrroles (Figure 4a). This is because hydrophobic pyrroles contributed more to browning than hydrophilic pyrroles.^{10,11} No significant ($P > 0.05$) changes in hydrophobic pyrrole content were found in AP dispersions during 32 days of storage at 2 °C, and therefore only data on day 0 are shown in Figure 4a. The observation that pyrrole content did not increase during storage was in agreement with the ³¹P NMR analysis, which also showed no decreases of PE and LPE due to the negligible PE pyrrolization in dispersions upon storage (Table 4). Furthermore, the hydrophobic pyrrole content increased in AP dispersions with increasing AP content from AP1 or APT to AP4 (Figure 4). As mentioned earlier, dispersions containing higher levels of AP (AP3 and AP4) or α -tocopherol (APT) showed a lower increment of volatiles after 32 days of storage; the relatively better oxidative stability in these dispersions could at least partly be attributed to the higher content of pyrroles in AP3 and AP4 dispersions or synergism between pyrroles and α -tocopherol as shown in APT dispersion. According to Hidalgo et al.,¹¹ antioxidative properties of pyrroles were greatly improved with the addition of α -tocopherol. In other words, the pyrroles that were present in AP dispersion could exhibit protective effects against oxidation.

To study the color changes induced by the pyrrolization, browning development in marine PL dispersions was determined by measurement of lightness (L^*) and yellowness index (YI). As suggested in our previous study,¹⁴ two types of pyrroles could be produced during the pyrrolization process in dispersions containing an amine group, namely, N-substituted pyrroles, which are stable, and 2-(1-hydroxyalkyl)pyrroles, which are unstable. 2-(1-Hydroxyalkyl)pyrroles could be further polymerized to form pyrroles in polymer form that were responsible for browning development.³³ However, it

cannot be ruled out that the polymerization of lipid oxidation products also produced brown oxypolymers that give additional color to AP dispersions.³⁴ No significant ($P > 0.05$) change in YI was found in AP dispersions during 32 days of storage at 2 °C, and therefore only data on day 0 are shown in Figure 4b,c. In addition, due to the high initial content of pyrroles in AP raw materials, the color changes of marine PL dispersions upon storage were difficult to observe. However, color differences between the different formulations of AP dispersions could easily be observed. AP1 and APT dispersions were expected to have higher lightness and lower YI than other dispersions as AP1 and APT contained lower percentages of AP. Surprisingly, a higher YI was observed in AP1 and APT dispersions as compared AP2–AP4 dispersions (Figure 4b,c). This phenomenon was due to the decrease in b^* (yellowness/blueness) and lightness (L^*) as the brownness increased in AP2–AP4 dispersions as observed visually.

Role of α -Tocopherol in Lipid Oxidation and Non-enzymatic Browning. As shown in Table 2, untreated marine PL had an induction period of 1500 min due to the presence of natural antioxidant (73.4 μ g/g of α -tocopherol). Its induction period decreased drastically to 41 min after purification due to the removal of α -tocopherol. As expected, addition of α -tocopherol (600 mg/kg) to purified marine PL significantly extended its induction period, and the end of the induction was not attained, at least not during the time period studied. In addition, both PV and volatiles data also showed that dispersion APT (containing α -tocopherol) had higher oxidative stability as compared to dispersion AP1 despite their similar lipid contents (Figures 1 and 2a,b). The above-mentioned results confirmed that tocopherol is an efficient antioxidant in PL dispersions. Several studies^{6,9} reported that the synergistic effect of PL on the antioxidant activity of α -tocopherol might contribute to the

high oxidative stability of marine PL. This phenomenon is most likely due to the hydrogen transfer from the amine group of PE to tocopheroxyl radical and regeneration of tocopherol or the secondary antioxidant action of PE in reducing quinines formed during oxidation of tocopherols. In addition, the synergism between α -tocopherol and pyrroles might also contribute to the high oxidative stability of marine PL.¹¹ APT dispersion was prepared from 0.25 mg of α -tocopherol per gram of PL (equal to 5 mg of α -tocopherol per kg of dispersion), and a small proportion of α -tocopherol was destroyed during the dispersion preparation step itself as the initial content of α -tocopherol in APT on 0 day was <5 mg/kg. The content of α -tocopherol in APT slightly decreased after 32 days of storage, from 3.41 mg/kg on 0 day to 2.64 mg/kg on day 32, as it was consumed due to lipid oxidation (data not shown). In terms of oxidized lipid-amine products, dispersion prepared from purified marine PL with addition of α -tocopherol (APT) also had the lowest content of 3-methylbutanal (Figure 2c). Both AP1 and APT had similar levels of lipids, but the level of Strecker aldehydes was much higher in AP1 than in APT. This was most likely due to the decrease of lipid oxidation in APT dispersion after addition of α -tocopherol and subsequently also led to a decrease in SD. In general, addition of α -tocopherol to purified marine PL dispersions decreased both lipid oxidation and oxidized lipid-amine reaction, namely, Strecker degradation.

AUTHOR INFORMATION

Corresponding Author

*Phone: +45 45 25 25 59. Fax: +45 45 88 47 74. E-mail: chja@food.dtu.dk.

Notes

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REFERENCES

- (1) Miyashita, K.; Nara, E.; Ota, T. Comparative study on the oxidative stability of phosphatidylcholines from salmon egg and soybean in an aqueous solution. *Biosci., Biotechnol., Biochem.* **1994**, *58*, 1772–1775.
- (2) Boyd, L. C.; Nwosu, V. C.; Young, C. L.; MacMillan, L. Monitoring lipid oxidation and antioxidant effects of phospholipids by headspace gas chromatographic analyses of rancimat trapped volatiles. *J. Food Lipids* **1998**, *5*, 269–282.
- (3) Mozuraityte, R.; Rustad, T.; Sorro, I. Pro-oxidant activity of Fe²⁺ in oxidation of cod phospholipids in liposomes. *Eur. J. Lipid Sci. Technol.* **2006**, *108*, 218–226.
- (4) Mozuraityte, R.; Rustad, T.; Sorro, I. Oxidation of cod phospholipids in liposomes: effects of salts, pH and zeta potential. *Eur. J. Lipid Sci. Technol.* **2006**, *108*, 944–950.
- (5) Mozuraityte, R.; Rustad, T.; Sorro, I. The role of iron in peroxidation of polyunsaturated fatty acids in liposomes. *J. Agric. Food Chem.* **2008**, *56*, 537–543.
- (6) Moriya, H.; Kunimino, T.; Hosokawa, M.; Fukunaga, K.; Nishiyama, T.; Miyashita, K. Oxidative stability of salmon and herring roe lipids and their dietary effect on plasma cholesterol levels of rats. *Fish. Sci.* **2007**, *73*, 668–674.
- (7) Lu, F. S. H.; Nielsen, N. S.; Timm-Heinrich, M.; Jacobsen, C. Oxidative stability of marine phospholipids in the liposomal form and their applications. *Lipids* **2011**, *46*, 3–23.
- (8) Applegate, K. R.; Glomset, J. A. Computer-based modeling of the conformation and packing properties of docosahexaenoic acid. *J. Lipid Res.* **1986**, *27*, 658–680.

(9) Cho, S. Y.; Joo, D. S.; Choi, H. G.; Nara, E.; Miyashita, K. Oxidative stability of lipids from squid tissues. *Fish. Sci.* **2001**, *67*, 738–743.

(10) Hidalgo, F. J.; Nogales, F.; Zamora, R. Changes produced in the antioxidative activity of phospholipids as a consequence of their oxidation. *J. Agric. Food Chem.* **2005**, *53*, 659–662.

(11) Hidalgo, F. J.; León, M. M.; Nogales, F.; Zamora, R. Effect of tocopherols in the antioxidative activity of oxidized lipid-amine reaction products. *J. Agric. Food Chem.* **2007**, *55*, 4436–4442.

(12) Bandarra, N. M.; Campos, R. M.; Batista, I.; Nunes, M. L.; Empis, J. M. Antioxidant synergy of alpha-tocopherol and phospholipids. *J. Am. Oil Chem. Soc.* **1999**, *76*, 905–913.

(13) Weng, X. C.; Gordon, M. H. Antioxidant synergy between phosphatidylethanolamine and α -tocopherylquinone. *Food Chem.* **1993**, *48*, 165–168.

(14) Lu, F. S. H.; Nielsen, N. S.; Baron, C.; Jacobsen, C. Oxidative degradation and non-enzymatic browning between oxidized lipids and primary amine groups in different marine PL dispersions. *Food Chem.* **2012**, *135*, 2887–2896.

(15) Schneider, M.; Lovaas, E. Process for the production of phospholipids. *US2009/0028989*, 2009.

(16) Lu, F. S. H.; Nielsen, N. S.; Baron, C.; Jensen, L. H. S.; Jacobsen, C. Physicochemical properties of marine phospholipid dispersions. *J. Am. Oil Chem. Soc.* **2012**, *89*, 2011–2024.

(17) AOCS Official Method Ce 8-89. Determination of tocopherols and tocotrienols in vegetable oils and fats by HPLC. In *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 5th ed.; AOCS: Champaign, IL, 1998.

(18) AOCS Official Method Ce 2-66. Preparation of methyl esters of long chain fatty acids. In *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 5th ed.; AOCS: Champaign, IL, 1998.

(19) AOCS Official Method Ce 1b-89. Fatty acids composition of marine oils by GLC. In *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 5th ed.; AOCS: Champaign, IL, 1998.

(20) International IDF Standard 74 A. Milk and milk products: determination of the iron content. International Dairy Federation: Brussels, Belgium, 1991.

(21) 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.

(22) AOCS Official Method Ce Sa-40. Free fatty acids. In *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 5th ed.; AOCS: Champaign, IL, 1998.

(23) Iverson, J. S.; Lang, L. C. S.; Cooper, M. H. Comparison of the Bligh and Dyer and Folch methods for total lipid determination in a broad range of marine tissue. *Lipids* **2001**, *36*, 1283–1287.

(24) Francis, F. J.; Clydesdale, F. H. *Food Colorimetry: Theory and Application*; AVI Publishing: Westport, CT, 1975.

(25) Peng, J. L.; Larondelle, Y.; Pham, D.; Ackman, R. G.; Rollin, X. Polyunsaturated fatty acid profiles of whole body phospholipids and triacylglycerols in anadromous and landlocked Atlantic salmon (*Salmo salar* L.) fry. *Comp. Biochem. Physiol., B: Comp. Biochem.* **2003**, *134*, 335–348.

(26) Grit, M.; Crommelin, D. J. A. Chemical stability of liposomes: implications for their physical stability. *Chem. Phys. Lipids* **1993**, *64*, 3–18.

(27) Grit, M.; Zuidam, N. J.; Underberg, W. J. M.; Crommelin, D. J. A. Hydrolysis of partially saturated egg phosphatidylcholine in aqueous liposome dispersions and the effect of cholesterol incorporation in hydrolysis kinetics. *J. Pharm. Pharmacol.* **1993**, *45*, 490–495.

(28) Saito, H.; Udagawa, M. Application of NMR to evaluate the oxidative deterioration of brown fish meal. *J. Sci. Food Agric.* **1992**, *58*, 135–137.

(29) Ventanas, S.; Estevez, M.; Delgado, C. L. Phospholipid oxidation, non-enzymatic browning development and volatile compounds generation in model systems containing liposomes from

porcine *Longissimus dorsi* and selected amino acids. *Eur. J. Lipid Sci. Technol.* **2007**, *225*, 665–675.

(30) Hidalgo, F. J.; Zamora, R. Strecker-type degradation produced by the lipid oxidation products 4,5-epoxy-2-alkenals. *J. Agric. Food Chem.* **2004**, *52*, 7126–7131.

(31) Zamora, R.; Gallardo, E.; Hidalgo, F. Strecker degradation of phenylalanine initiated by 2,4-decadienal or methyl 13-oxooctadeca-9,11-dienoate in model systems. *J. Agric. Food Chem.* **2007**, *55*, 1308–1314.

(32) Pripis-Nicolau, L.; Revel, G. D.; Bertrand, A.; Maujean, A. Formation of flavor components by the reaction of amino acid and carbonyl compounds in mild conditions. *J. Agric. Food Chem.* **2000**, *48*, 3762–3766.

(33) Hidalgo, F. J.; Nogales, F.; Zamora, R. Effect of the pyrrole polymerization mechanism on the antioxidative activity of non-enzymatic browning reactions. *J. Agric. Food Chem.* **2003**, *51*, 5703–5708.

(34) Khayat, A.; Schwall, D. Lipid oxidation in seafood. *Food Technol.* **1983**, *37*, 130–140.

PAPER V

Lu, F. S. H., Nielsen, N. S., Baron, C. P., Diehl, B. W. K., & Jacobsen, C.

Impact of primary amine group from aminophospholipids and amino acids on marine phospholipid stability: Non-enzymatic browning and lipid oxidation.

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Impact of primary amine group from aminophospholipids and amino acids on marine phospholipids stability: Non-enzymatic browning and lipid oxidation



F.S.H. Lu^a, N.S. Nielsen^a, C.P. Baron^a, B.W.K. Diehl^b, C. Jacobsen^{a,*}

^a Division of Industrial Food Research, Technical University of Denmark, Søtofts Plads, Building 221, 2800 Kgs. Lyngby, Denmark

^b Spectral Service AG, Emil-Hoffmann-Straße 33, D-50996 Köln, Germany

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ABSTRACT

The main objective of this study was to investigate the oxidative stability and non-enzymatic browning reactions of marine PL in the presence or in the absence of primary amine group from aminophospholipids and amino acids. Marine phospholipids liposomal dispersions were prepared from two authentic standards (phosphatidylcholine and phosphatidylethanolamine) and two purified PL from marine sources with and without addition of amino acids (leucine, methionine and lysine). Samples were incubated at 60 °C for 0, 2, 4 and 6 days. Non-enzymatic browning reactions were investigated through measurement of (i) Strecker derived volatiles, (ii) yellowness index (YI), (iii) hydrophobic and (iv) hydrophilic pyrroles content. The oxidative stability of the samples was assessed through measurement of secondary lipid derived volatile oxidation products. The result showed that the presence of PE and amino acids caused the formation of pyrroles, generated Strecker derived volatiles, decreased the YI development and lowered lipid oxidation. The lower degree of lipid oxidation in liposomal dispersions containing amino acids might be attributed to antioxidative properties of pyrroles or amino acids.

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1. Introduction

Marine phospholipids (PL) have received much attention recently due to their advantages as compared to fish oil in triglycerides (TAG) form and these advantages include: (a) a higher content of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Peng, Larondelle, Pham, Ackman, & Rollin, 2003); (b) a better bio-availability for EPA and DHA (Wijendran et al., 2002); (c) a better resistance towards oxidation due to the antioxidative properties of PL (Cho, Joo, Choi, Nara, & Miyashita, 2001; Moriya et al., 2007). Oxidative stability of marine PL especially in the form of emulsion or liposomal system has been reviewed extensively in our previous publication (Lu, Nielsen, Timm-Heinrich, & Jacobsen, 2011). The antioxidative properties of marine PL have been proposed to be a consequence of (a) their tight intermolecular packing conformation with the PUFA at the *sn*-2 position (Applegate & Glomset, 1986; Miyashita, Nara, & Ota, 1994) and (b) synergism between the phospholipids and α -tocopherol, which is also present in marine PL (Cho et al., 2001; Moriya et al., 2007); (c) protective effect exhibited by pyrroles, antioxidative compounds resulting

from non-enzymatic browning reactions between primary amine group of phosphatidylethanolamine/amino acids and lipid oxidation products in marine PL (Lu, Nielsen, Baron, & Jacobsen, 2012). Like in other food systems, lipid oxidation and non-enzymatic browning reactions are suggested to be important reactions in PL. These reactions follow parallel reaction pathways and constitute important deteriorative mechanisms that can cause significant changes in flavor, colour, texture and nutritional value of PL (Zamora & Hidalgo, 2005).

In fact, the non-enzymatic browning reaction resulting from oxidised lipids has gained considerable attention recently and was reviewed extensively by Zamora and Hidalgo (2005). Furthermore, several studies (Hidalgo, Mercedes leon, Nogales, & Zamora, 2007; Hidalgo, Mercedes leon, & Zamora, 2006; Hidalgo, Nogales, & Zamora, 2005a) have investigated the antioxidative properties of pyrroles formed in slightly oxidised soybean phosphatidylethanolamine (PE) or phosphatidylcholine (PC) and phosphatidylinositol (PI) after reaction with amino acids. However, information about pyrrrolisation and non-enzymatic browning reactions in more complex systems such as in marine PL dispersions such as liposome is scarce. Moreover, only few studies (Thanonkaew, Benjakul, Vises-sanguan, & Decker, 2006, 2007) investigated the pyrrrolisation or browning development in a marine PL liposome system. These studies provided no information about Strecker degradation (SD)

* Corresponding author. Tel.: +45 45252559; fax: +45 45884774.
 E-mail address: chja@food.dtu.dk (C. Jacobsen).

products, which are also formed as a result of non-enzymatic browning reactions. Therefore more comprehensive studies within this area are needed.

Our previous study showed that the content of α -tocopherol, initial hydroperoxides, pyrroles, transition metals, etc. could affect the oxidative stability of marine PL emulsions (Lu, Nielsen, Baron, & Jacobsen, 2012). In order to avoid the interference of above mentioned compounds toward lipid oxidation, two marine PL previously used were chosen for purification in the present study. The main objective of the present study was to investigate if the presence of primary amine group from PE or amino acids affects the formation of pyrroles and SD products and whether this in turn will affect the oxidative stability of purified marine PL liposomal dispersions. In addition to purified marine PL, pure PC and PE were used as reference for comparison to investigate the non-enzymatic browning development in liposomal dispersions. PC is the most dominant PL in purified marine PL whereas PE is the PL that usually involves in pyrrolisation as previously mentioned and therefore these two PL were included in our experimental design. Furthermore, a molecule species comprising a palmitic acid (PA) at *sn*-1 position and a docosahexaenoic acid (DHA) at *sn*-2 position was chosen for both PC and PE as this is one of the most dominant molecule species in marine PL (Le Grandois et al., 2009). Lysine, leucine and methionine were chosen as the source of primary amine as they are previously reported to generate abundant Strecker derived volatiles in marine PL emulsions (Lu, Nielsen, Baron, & Jacobsen, 2012). In addition, lysine is a reactive amino acid residue that usually is involved in both carbohydrate/protein and oxidised lipid/protein reactions.

During the pyrrole formation process, we studied the changes in yellowness index (YI) and volatile profile of purified and isolated marine PL liposomal dispersions in comparison to liposomal dispersions prepared from authentic standards of PE and PC. In addition, we determined hydrophilic and hydrophobic pyrroles formed. In general, this study provided an improved understanding of the mechanism for non-enzymatic browning reactions in marine PL liposomal dispersions.

2. Materials

Two different marine phospholipids (LC and MPW) were obtained from PhosphoTech Laboratoires (Saint-Herblain Cedex, France) and Triple Nine (Esbjerg, Denmark), respectively. MPW comprised 41.50% of total PL, 18.30% PC, 4.70% PE, 2.10% PI, 3.40% LPC and 8.9% other PL. LC comprised 43.84% of total PL, 20.87% PC, 6.11% PE, 0.96% PI, 1.59% SPM and 3.57% LPC. In terms of fatty acids composition, MPW comprised 27.30% of C16:0, 14.10% of C18:1, 12.53% of C20:5 and 32.8% of C22:6, whereas LC comprised 28.20% of C16:0, 3.22% of C18:1, 14.89% of C20:5 and 40.03% of C22:6. Two synthetic PL (PC and PE) standards were purchased from Avanti Polar Lipids (Alabama, USA). Both PE and PC standards had purity >99% and contained C16:0 fatty acids at *sn*-1 position and C22:6 fatty acids at *sn*-2 position.

3. Methods

3.1. Purification of marine PL

Marine PL (2 g) were extracted with 10 mL of chloroform-methanol (1:1) with addition of 5 mL of distilled water. The resulting organic layer was further diluted with chloroform to obtain a final solution of 20 mL prior to separation by Solid Phase Microextraction (SPE) according to an adapted method from Kim and Salem (1990). Approximately 5 mL of diluted marine PL in chloroform was transferred to a Sep-pak column containing 10 g

aminopropyl-modified silica (Waters Corporation, Milford, Massachusetts, USA) for lipid separation. A mixture of 2 × 10 mL chloroform and 2-propanol (ratio 2:1) was used to elute the neutral lipid fraction (NL) whereas a mixture of 3 × 10 mL diethyl ether and acetic acid (ratio 98:2) was used to elute free fatty acids (FFA) and finally a mixture of 3 × 10 mL methanol was used to elute the PL fraction by gravity. This separation procedure was repeated 4 times for (4 × 5 mL) diluted marine PL chloroform. The NL and FFA fractions were discarded whereas the PL fractions were pooled together and evaporated under nitrogen until dryness.

3.2. Preparation of model marine PL liposomal dispersion

Approximately 500 mg PC or PE standard or purified marine PL was dissolved in 150 mL of sodium phosphate buffer (50 mM, pH 7). The solution was then sonicated using a sonicator (Branson 2150E-MT, Branson Ultrasonics Corporation, CT, USA, with alimention: 220–230 V, 50–60 Hz) for approximately 45 min at room temperature until a homogenous dispersion was obtained. The final solution was divided into 2 × 75 mL blue capped bottles and a mixture of amino acids comprising lysine, leucine and methionine (100 mg of each) was added into one of the bottles (75 mL) as shown in Table 1. All samples were incubated at 60 °C. Samples were taken on day 0, 2, 4, 6 days and flushed with nitrogen and stored at –40 °C until further analysis. Samples were analysed for oxidative stability by measuring lipid derived volatiles through Solid Phase Microextraction (SPME) GC–MS. In addition, Strecker derived volatiles were measured using the same method in order to study non-enzymatic browning reactions in marine PL samples. The investigation of non-enzymatic browning reactions included also the measurement of yellowness index (YI) and pyrrole content in marine PL liposomal dispersions. PL can spontaneously self-assemble and form liposomes in the presence of water. Therefore, the dispersion prepared from PE and two purified marine PL in this study were found to contain mainly liposome of average diameter 0.1 µm, as also reported in our previous study (Lu, Nielsen, Baron, Jensen, & Jacobsen, 2012), whereas PC dispersion contained liposome of average diameter approximately 5 µm. Therefore, the PL dispersions prepared in this study were called as liposomal dispersions.

3.3. Headspace analysis using solid phase microextraction (SPME) GC–MS

Approximately 1 g of sample, together with 30 mg of internal standard (10 µg/g of 4-methyl-1-pentanol in rapeseed oil) was mixed on a whirlly mixer for 30 s in a 10 mL vial. The sample was equilibrated for 3 min at a temperature of 60 °C, followed by extraction for 45 min at the same temperature while agitating the sample

Table 1
Experimental design for PL liposomal dispersions.

Liposomal dispersions ^a	Amino acids (mg)			Concentration of amino acids (mg/mL)
	Lysine	Leucine	Methionine	
DPC	–	–	–	
DPCA	100	100	100	1.33
DPE	–	–	–	
DPEA	100	100	100	1.33
DLC	–	–	–	
DLCA	100	100	100	1.33
DMPW	–	–	–	
DMPWA	100	100	100	1.33

^a DPE and DPC are dispersions prepared from authentic standards phosphatidylcholine and phosphatidylethanolamine; DLC and DMPW are dispersions prepared from purified marine PL (LC and MPW); DPEA, DPCA, DLCA and DMPWA are dispersions added with amino acids, namely leucine, methionine and lysine.

at 500 rpm. Extraction of headspace volatiles was done by 50/30 µm CAR/PDMS SPME fibre (Supelco, Bellefonte, PA, USA) installed on a CTC Combi Pal (CTC Analytics, Waldbronn, Germany). Volatiles were desorbed in the injection port of gas chromatograph (HP 6890 Series, Hewlett Packard, Palo Alto, CA, USA; Column: DB-1701, 30 m × 0.25 mm × 1.0 µm; J&W Scientific, CA, USA) for 60 s at 220 °C. The oven program had an initial temperature of 35 °C for 3 min, with increment of 3.0 °C/min to 140 °C, then increment of 5.0 °C/min to 170 °C and increment of 10.0 °C/min to 240 °C, where the temperature was held for 8 min. The individual compounds were analysed by mass-spectrometry (HP 5973 inert mass-selective detector, Agilent Technologies, USA; Electron ionisation mode, 70 eV, mass to charge ratio scan between 30 and 250). In order to investigate lipid oxidation in marine PL liposomal dispersions, the following *n*-3 derived secondary volatiles were selected for quantification by abundance values obtained from the MS analysis: propenol, 2-ethylfuran, 1-penten-3-one, *E*-2-hexenal, *Z*-4-heptenal, *E,E*-2,4-heptadienal and *E,Z*-2,6-nonadienal. Measurements were made in triplicates on each sample. SPME GC–MS analysis was also used for identification of Strecker derived volatiles.

3.4. Measurement of yellowness index (YI) and pyrrole content

PL liposomal sample (3 mL) was extracted twice with 6 mL of chloroform–methanol (2:1) and the resulting organic and aqueous extracts were analysed for yellowness index (YI) and pyrrole content. Organic extract (0.5 g) was dried under nitrogen and 1 mL of 150 mM sodium phosphate (pH 7) containing 3% sodium dodecyl sulfate (SDS) was added. This solution was then treated with Ehrlich reagent (700 µL of reagent A and 170 µL of reagent B). Reagent A was prepared by mixing 2 mL ethanol with 8 mL HCl (2.5 N) while reagent B was prepared by suspending 200 mg of *p*-(dimethylamino)benzaldehyde in 10 mL of reagent A. The final solution was incubated at 45 °C for 30 min. The absorbance of the maximum at 570 nm was measured against a blank prepared under the same conditions but without *p*-(dimethylamino)benzaldehyde. Aqueous extracts (1 mL) was analysed using the same method without further treatment. Two extractions were made from each sample and the measurement was performed in duplicate. Pyrroles content was quantified by an authentic external standard, 1-(4-methoxyphenyl)-1H-pyrrole (this standard give absorbance at 570 nm). The pyrrole concentration is thus given as mmol 1-(4-methoxyphenyl)-1H-pyrrole/g sample. Colour changes were measured on the organic extract as well using a spectrophotometer (X-Rite, Inc. Grandville, MI, USA). The instrument was calibrated before each measurement and the results were recorded using the CIE colour system profile of L^* (Lightness), a^* (redness/greenness), b^* (yellowness/blueness). In addition, yellowness index (YI) was calculated according to Francis and Clydesdale (1975): $YI = 142.86 b^*/L^*$. Two extractions were performed on each sample and the measurement was performed in duplicate.

3.5. Determination of amino acids composition

PL liposomal sample (3 mL) was extracted twice with 6 mL of chloroform–methanol (2:1) and the resulting aqueous extract (methanol–water phase) was analysed for amino acids content by EZ:faast Hydrolysate Amino Acids Analysis kit (Phenomenex, CA, USA). A summary of procedure according to the user's manual EZ:faast is stated as follows: One hundred microlitres of marine PL aqueous extract, 100 µL of internal standard (homoarginine 0.2 mM, methionine- d_3 0.2 mM and homophenylalanine 0.2 mM) were combined in a glass vial and mixed by two short bursts on a vortex mixer. An ion exchange resin solid phase extraction (SPE) tip was attached to a 1.5 mL syringe and the solution was pulled slowly through to completion. Two hundred microlitres of

wash solution (water) was added to the glass vial and also pulled slowly through the SPE tip to completion. The 1.5 mL syringe was removed while leaving the SPE tip inside the glass vial. Two hundred microlitres of a premixed elution buffer (sodium hydroxide and *n*-propanol) was then added to the glass vial. The piston of a 0.6 mL syringe was pulled halfway up the barrel and the syringe was attached to the SPE tip. Elution buffer was drawn into the SPE tip and stopped when the buffer reached the filter plug in the SPE tip. Both the buffer and the sorbent material were quickly expelled out from the tip into the glass vial. This step was repeated until all of the material had been expelled. Fifty microlitres of derivatising reagent, chloroform was added to the glass vial and the mixture was vortexed vigorously for 8 s. The solution was allowed to react for 1 min and the vortexing step repeated. One hundred microlitres of organic reagent, iso-octane was then added to the sample and vortexed vigorously for 5 s. The mixture was allowed to stand for 1 min for phase separation. After 1 min of the phase separation, 150 µL of the upper organic layer was taken, dried under nitrogen and redissolved with 100 µL of methanol:water (2:1) prior to analysis by LC/MS system (Agilent 1100 series, Agilent Technologies, Palo Alto, CA, USA; column: EZ:faast AAA-MS column 250 × 3.0 mm). The mobile phases consisted of A: 10 mM Ammonium formate in water, B: 10 mM Ammonium formate in methanol and was introduced at a flow rate of 0.5 mL/min. Gradient used: 20 min for 83% B, 20.01 min for 60% B, followed by 26 min for 60% B. The individual compounds were analysed by mass-spectrometry (APCI, positive mode, scan range: 100–600 *m/z*, APCI ionisation chamber temperature of 450 °C).

3.6. Measurement of PE losses and PL hydrolysis (P NMR)

PE and also other PL content of marine PL was determined through ^{31}P NMR by Spectra Service GmbH (Cologne, Germany). All spectra were acquired using NMR spectrometer Avance III 600 (Bruker, Karlsruhe, Germany), magnetic flux density 14.1 Tesla QNP cryo probe head and equipped with automated sample changer Bruker B-ACS 120. Computer Intel Core2 Duo 2.4 GHz with MS Windows XP and Bruker TopSpin 2.1 was used for acquisition, and Bruker TopSpin 2.1 was used for processing. Only single measurement was made with 5% detection limit.

3.7. Statistical analysis

The obtained data, volatiles, YI and pyrrole measurement were subjected to one way ANOVA analysis and comparison among samples were performed with Tukey multiple comparison test using a statistical package program Minitab 16 (Minitab Inc., State College, PA, USA). Significant differences were accepted at ($p < 0.05$). Multivariate analysis was performed by the Unscrambler (Unscrambler X, version 10.2, CAMO Software AS, Oslo, Norway). The main variance in the data set was studied using principal component analysis. The data set included variables of non-enzymatic browning reactions: yellowness index (YI), Strecker derived volatiles, PE losses, hydrophobic and hydrophilic pyrroles and variables of lipid oxidation included *n*-3 derived volatiles. All data were centred and auto-scaled (1/standard deviation) to equal variance prior to PCA analysis.

4. Results and discussion

4.1. Investigation of non-enzymatic browning reactions

4.1.1. Strecker degradation

Strecker degradation (SD) of amino acids involves the oxidative deamination of α -amino acids in the presence of compounds such

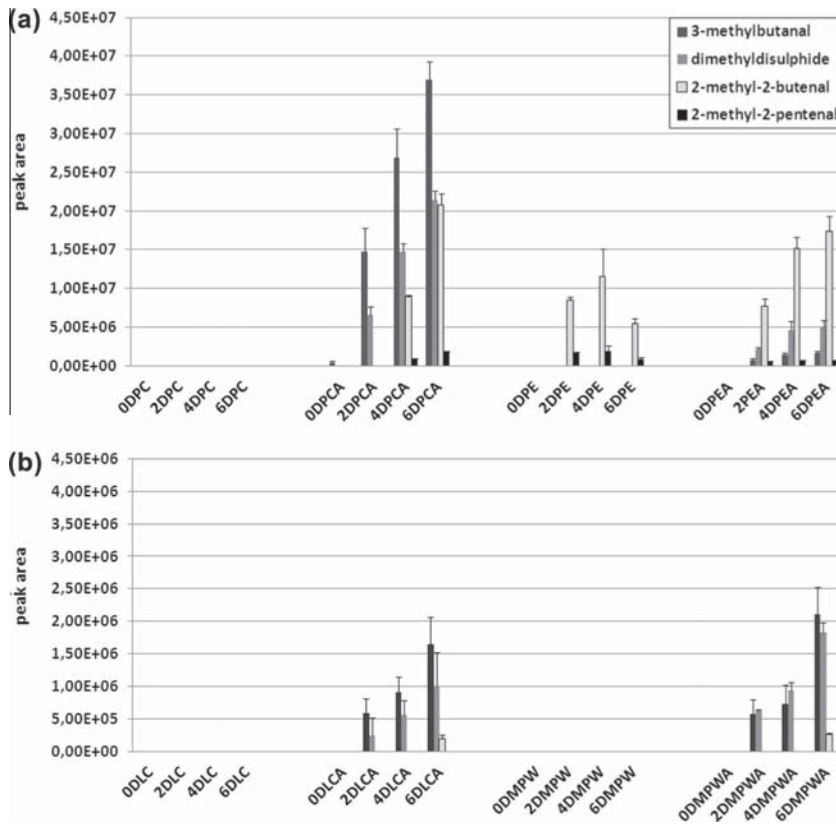


Fig. 1. Measurement of Strecker derived volatiles in liposomal dispersions over 6 days incubation at 60 °C. DPE and DPC are dispersions prepared from authentic standards phosphatidylcholine and phosphatidylethanolamine; DLC and DMPW are dispersions prepared from purified marine PL (LC and MPW); DPEA, DPCA, DLCA and DMPWA are dispersions added with amino acids, namely leucine, methionine and lysine. Values are means \pm standard deviation ($n = 3$).

as reducing sugars or some lipid oxidation products (Zamora & Hidalgo, 2011). In this study, SD occurred mainly between amine group from amino acids or PE with lipid oxidation product. As shown in Fig. 1, Strecker derived volatiles were detected primarily in liposomal dispersions containing a primary amine group, namely DPCA, DPE, DPEA, DLCA and DMPWA, but not in DPC nor in the liposomal dispersions prepared with purified marine PL without amino acids (LC or MPW), which mainly contained PC. Strecker derived volatiles such as 3-methylbutanal, dimethylsulphide, 2-methyl-2-pentenal and 2-methyl-2-butenal increased ($p < 0.05$) in DPCA over 6 days incubation at 60 °C. The same observation was obtained for DPEA with amino acids added. It has previously been suggested that 3-methylbutanal, dimethylsulphide from a reaction with tertiary lipid oxidation products whereas dimethylsulphide was found to be a degradation product of methionine (Ventanas, Estevez, & Delgado, 2007). In addition, 2-methyl-2-pentenal and 2-methyl-2-butenal were suggested to be the major volatiles resulting from a reaction between tertiary lipid oxidation products originating from (*E,E*)-2,4-heptadienal with lysine (Zamora, Rios, & Hidalgo, 1994). According to the mechanism suggested by Zamora et al. (1994) in a model system consisting of (*E,E*)-2,4-heptadienal with lysine, 2-methyl-2-pentenal could be produced by an aldol condensation between two molecules of propanal, whereas 2-methyl-2-butenal could be produced from

one molecule of propanal and one molecule of acetaldehyde, which was previously degraded from propanal. However, 2-methyl-2-pentenal and 2-methyl-2-butenal were also found in DPE after incubation and this might be attributed to the reaction between tertiary lipid oxidation products with primary amine group of PE. Furthermore, the involvement of amino acids in Strecker degradation in the present study was confirmed by analysis of amino acids left in samples after incubation and the percentage of amino acids losses over time was more pronounced for leucine > methionine (data not shown).

It is suggested that these Strecker derived volatiles were produced via reaction between amino acids with tertiary lipid oxidation products such as unsaturated epoxy keto fatty esters, epoxyalkenals and hydroxyalkenals (Fig. 2). According to Hidalgo and Zamora (2004), the presence of two oxygenated function groups in the tertiary lipid oxidation products, namely one carbonyl group and one epoxy or hydroxyl group is required for the SD reaction to occur as shown in mechanism A in Fig. 2. The tertiary lipid oxidation products are formed from secondary oxidation products such as alkadienals and ketodiene (Zamora, Gallardo, & Hidalgo, 2007). The increase in concentration of the Strecker derived volatiles in liposomal dispersions upon storage might be due to the increase of lipid oxidation. Furthermore, our previous findings (Lu et al., 2012a) showed that SD reaction occurred in

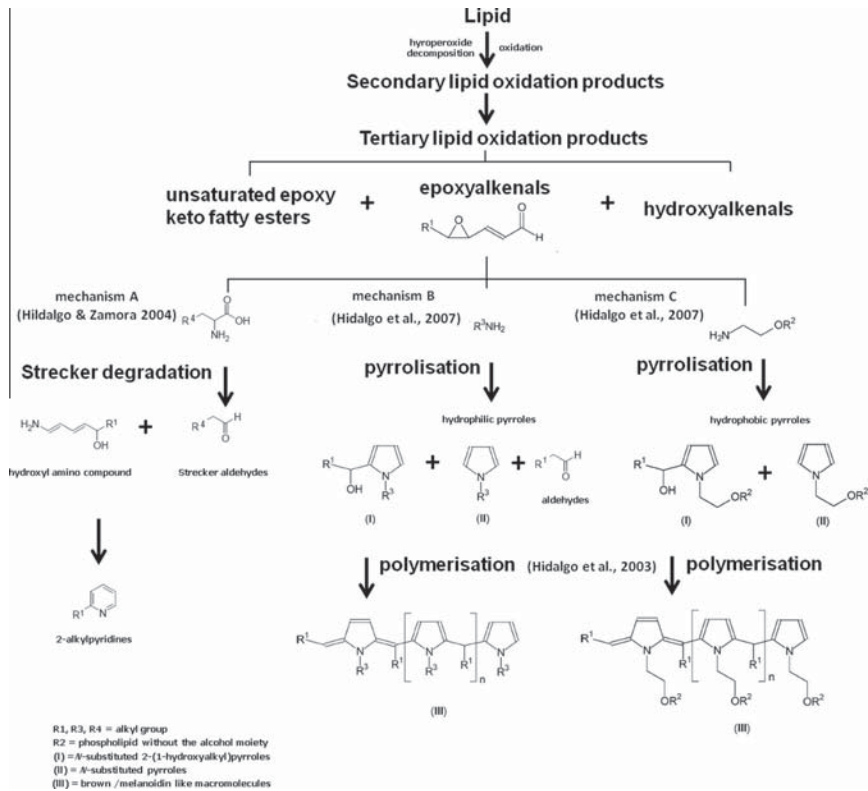


Fig. 2. Proposed mechanisms for non-enzymatic browning reactions in PL liposomal dispersions. Printed from Lu et al. (2012a) with permission from Elsevier.

marine PL emulsions during storage in parallel to lipid oxidation reaction.

Interestingly, less SD was observed with the detection of only three Strecker derived volatiles, namely 3-methylbutanal, dimethylsulphide and 2-methyl-2-pentenal in purified marine PL liposomal dispersions containing amino acids (DLCA and DMPWA) over 6 days incubation when compared to DPC and DPE (Fig. 1b). This phenomenon might be attributed to a higher degree of unsaturation in both PC and PE, which contain only one type of molecule species with a palmitic acid (PA) at *sn*-1 position and a docosahexaenoic acid (DHA) at *sn*-2 position, whereas phospholipids in purified marine PL contain several molecular species and not all of them having DHA at *sn*-2 position. The SD in liposomal dispersions prepared from authentic PC and PE was in the order: DPCA > DPEA > DPE, whereas for liposomal dispersions prepared from purified marine PL, the SD was greater in DMPWA than DLCA. This phenomenon might be due to the higher degree of lipid oxidation in former liposomal dispersions than the later liposomal dispersions.

4.1.2. Yellowness index (YI) and pyrrolisation in PE and PC liposomal dispersions

In order to further investigate the non-enzymatic browning reactions in PL liposomal dispersions, the development of yellowness index (YI) and pyrroles formation were followed over 6 days incubation at 60 °C. YI was measured as function of incubation time in organic layer of liposomal dispersions due to an appreciable browning development in this layer, which was not observed in aqueous layer. It is speculated that amine group pyrrolisation may

partly account for the occurrence of non-enzymatic browning development in DPE, DPEA and DPCA as illustrated by the formation of colour as shown by yellowness index, YI (Fig. 3a), hydrophobic pyrroles (Fig. 3b) and hydrophilic pyrroles (Fig. 3c). As proposed in our previous study (Lu et al., 2012a), non-enzymatic browning may originate from the reaction between reactive carbonyls originating from tertiary or secondary lipid oxidation products with the primary amine group from PE or amino acids added into the liposomal dispersions (Fig. 2). As shown by mechanism C (Fig. 2), if the pyrrolisation takes place between tertiary lipid oxidation products with free amine group present in PE, the pyrroles produced is likely to be hydrophobic. This hypothesis was confirmed by our experiment, where formation of hydrophobic pyrroles only in DPE and DPEA (Fig. 3b) was attributed to PE pyrrolisation as also showed by the decrease of PE content after 6 days of incubation (Fig. 3d). On the other hand, if the reaction takes place with amine group of amino acids (mechanism B in Fig. 2), the pyrroles produced may be more hydrophilic. This is further confirmed by our data showing formation of hydrophilic pyrroles only in DPCA and DPEA (Fig. 3c).

As also shown in proposed mechanism (Fig. 2), two types of pyrroles could be produced during the pyrrolisation process, namely N-substituted pyrroles which are stable and 2-(1-hydroxyalkyl)pyrroles, which are unstable. 2-(1-hydroxyalkyl)pyrroles could be further polymerized to form pyrroles in dimer or polymer form (Hidalgo & Zamora, 1993; Hidalgo et al., 2007). In fact, pyrroles formation and polymerisation are the processes responsible for the yellow colour or browning

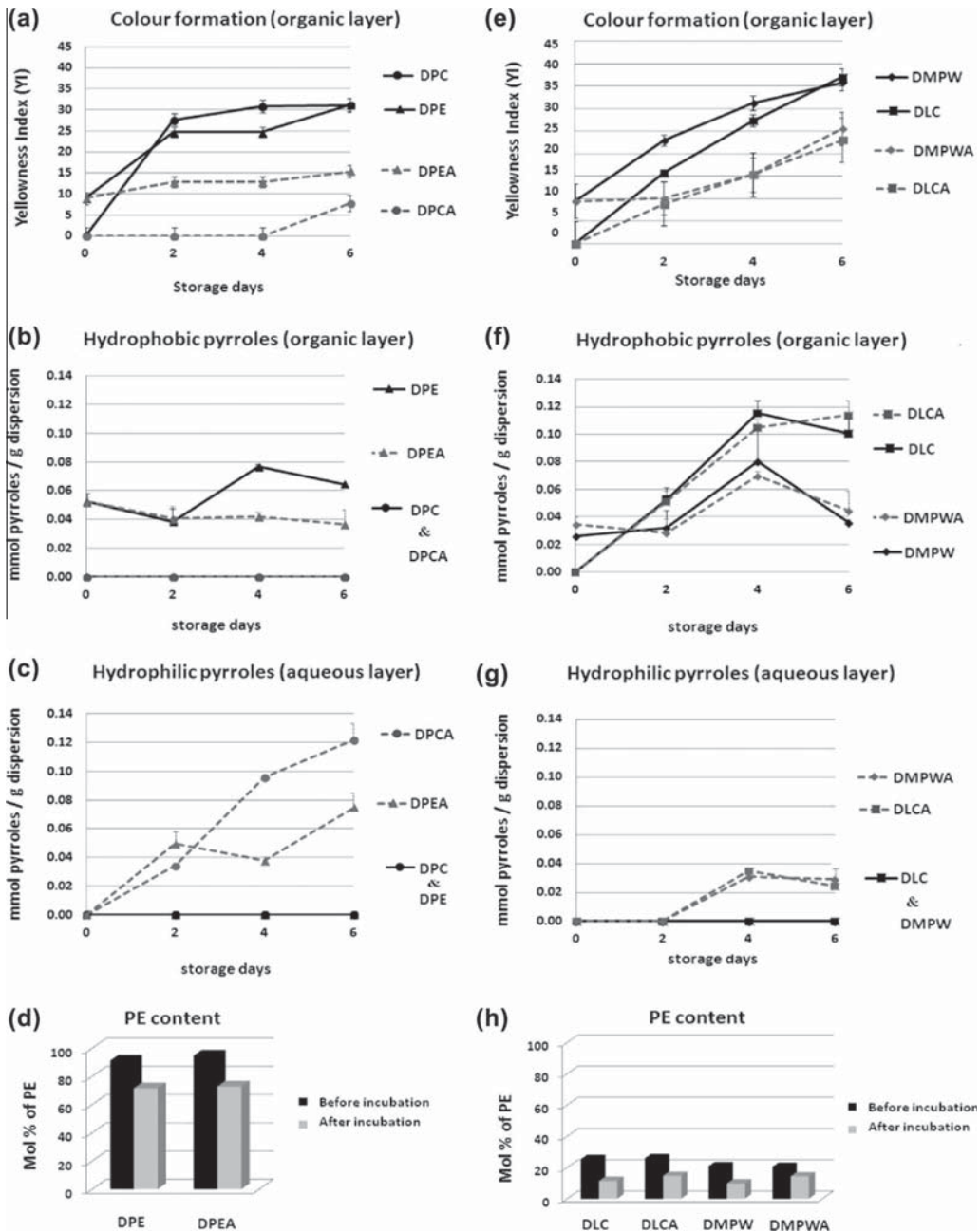


Fig. 3. Measurement of colour formation (a and e); hydrophobic pyrroles (b and f); hydrophilic pyrroles (c and g) and PE content (mol.%) (d and h) in liposomal dispersions over 6 days incubations at 60 °C. DPE and DPC are dispersions prepared from authentic standards phosphatidylcholine and phosphatidylethanolamine; DLC and DMPW are dispersions prepared from purified marine PL (LC and MPW); DPEA, DPCA, DLCA and DMPWA are dispersions added with amino acids, namely leucine, methionine and lysine. Values are means \pm standard deviation ($n = 2$), except for d and h, where a single measurement was made with 5% detection limit.

development in the liposomal dispersions containing amine group (Zamora, Alaiz, & Hidalgo, 2000). In addition, several studies (Hidalgo, Nogales, & Zamora, 2005b; Zamora, Nogales, & Hidalgo, 2005) reported that a correlation among yellow colour,

fluorescence and pyrroles measurement was observed in model system containing PE and amino acids.

In terms of colour formation in DPE, YI already developed in DPE at day 0 (approximately 10) and this may indicate that the

PE pyrrolisation occurred during the dispersion preparation step itself (Fig. 3a). This was most probably due to the close proximity of amine group of PE and the place of generation of lipid oxidation products and this increased the PE pyrrolisation (Zamora et al., 2000). YI increased drastically ($p < 0.05$) in DPE at 2 days of incubation and gradually increased thereafter (Fig. 3a). In terms of hydrophobic pyrroles formation (Fig. 3b), it significantly ($p < 0.05$) increased from day 2 to a maximum value at day 4 and decreased again thereafter (but not statistically significant). The decrease of pyrroles content especially at 6 days incubation is likely to be a consequence of PE oxidation. This is because the pyrrole as an antioxidant compound in the dispersion was consumed during the lipid oxidation (Hidalgo, Nogales, & Zamora, 2004). In fact, the antioxidative property of pyrroles has been reported in several studies (Hidalgo, Nogales, & Zamora, 2003; Hidalgo et al., 2005a, 2006, 2007). However, a longer incubation time is needed in future to further confirm the significant consumption of pyrroles during lipid oxidation.

Similar to DPE, YI increased significantly ($p < 0.05$) in DPC from 0 at day 0 to 27 at 2 days of incubation and gradually increased thereafter (Fig. 3a). It is conceivable that this browning was not due to amine group pyrrolisation as no pyrroles were found in this liposomal dispersion over 6 days of incubation (Fig. 3b and c). This observation supported the finding of Hidalgo et al. (2006), who also reported that no pyrrolisation was observed in soybean PC after incubation at 60 °C. It is most likely that brown coloured oxypolymers produced via the oxypolymerisation may account for the browning development in DPC. This is also largely explained by the fact that PC molecules contain highly unsaturated fatty acid, docosahexaenoic acid (DHA), which is highly susceptible to oxidation and thus led to oxypolymerisation of lipid oxidation products generated from this fatty acid (Khayat & Schwall, 1983). According to Uematsu et al. (2002), the increase in degree of unsaturation also led to the increase in non-enzymatic-browning reactions.

Furthermore, when a comparison was made between the YI development behaviour in both DPC and DPE, it seemed that the browning development in DPC was significantly ($p < 0.05$) faster than DPE during 6 days incubation. Similar to the PC molecule, every PE molecule contains a DHA and therefore it was speculated that both oxypolymerisation and pyrrolisation were responsible for the browning development in DPE, whereas only oxypolymerisation was responsible for browning development in DPC. Thus, it may be concluded that oxypolymerisation reaction was faster than pyrroles formation and polymerisation. This observation is in accordance with that of Zamora et al. (2000), who reported that browning development was much faster in fatty acid/lysine emulsion than alkenal/lysine emulsion involving only pyrroles formation and polymerisation.

For DPEA, YI tended to increase gradually (but not statistically significant) as incubation progressed from 0 to 6 days (Fig. 3a). This phenomenon might be due to occurrence of two types of pyrrolisation reactions in this liposomal dispersion as shown by formation of hydrophobic and hydrophilic pyrroles in Fig. 3b and c, respectively. It is assumed that the primary amine group from PE or amino acids were competing with each other to react with lipid oxidation products in the pyrrolisation process and thus decreased the lipid oxidation products that were available for oxypolymerisation and therefore less browning was observed. In terms of pyrroles content, the hydrophobic pyrroles in DPEA remained constant over time (the decrement was not significantly different) as shown in Fig. 3b, whereas hydrophilic pyrroles in DPEA increased gradually ($p < 0.05$) over time (Fig. 3c).

For DPCA, there was no browning development this liposomal dispersion initially, the appreciable browning ($p < 0.05$) was only observed at 6 days incubation (Fig. 3a). On the other hand, hydrophilic pyrroles content increased linearly ($p < 0.05$) in DPCA start-

ing from day 0 to day 6 of incubation (Fig. 3c). The formation of hydrophilic pyrroles was observed earlier than the browning development in DPCA (Fig. 3c). Taken together, it seems that pyrroles formation and certain level of polymerisation were required prior to browning development. This is in agreement with proposed mechanism that the reaction between tertiary lipid oxidation products with amine group produced in a first step both *N*-substituted pyrroles and 2-(1-hydroxyalkyl)pyrroles and followed by polymerisation of 2-(1-hydroxyalkyl)pyrroles, which were responsible for the colour development as mentioned earlier (Zamora et al., 2000). In general, the browning development in DPE and DPC was two to three times higher than liposomal dispersions with amino acids added (DPEA and DPCA). The non-enzymatic browning development was in the order: DPC > DPE > DPEA > DPCA. Hence, addition of amino acids into the liposomal dispersions decreased the browning development. This phenomenon might be due to antioxidative properties of pyrroles as mentioned earlier or the antioxidative properties of methionine and leucine (Chen, Muramoto, Yamauchi, & Nokihara, 1996; Guo, Kouzuma, & Yonekura, 2009), especially a high antioxidative effect of methionine has been demonstrated in several studies (Elias, McClements, & Decker, 2005; Levine, Mosoni, Berlett, & Stadman, 1996).

4.1.3. Yellowness index (YI) and pyrrolisation in purified marine PL liposomal dispersions

Analogous to DPC and DPE, non-enzymatic browning development was observed in liposomal dispersions prepared from purified marine PL as incubation progressed (Fig. 3e–h). For both DMPW and DLC, YI increased linearly ($p < 0.05$) in liposomal dispersions over time (Fig. 3e). Due to the presence of both PC and PE in purified marine PL (approximately 51% PC and LPC and 20% PE in MPL, 45% PC and LPC and 26% PE in LC as determined by ^{31}P NMR), it was speculated that browning in purified marine PL liposomal dispersions was due to two mechanisms (oxypolymerisation and PE pyrrolisation) as mentioned earlier. It was most likely that oxypolymerisation contributed more to browning development than PE pyrrolisation as PC content was much higher than PE in both purified marine PL. In addition, YI was higher in DMPW than DLC initially as purification method in this study did not remove all the yellow colour compounds that were present in neat MPW (YI = 10 in DMPW and YI = 0 in DLC), but after incubation the differences were ambiguous. In general, browning development rate was slightly faster ($p < 0.05$) in DLC than DMPW as incubation progressed. This interpretation was confirmed by a significantly ($p < 0.05$) higher hydrophobic pyrroles content in DLC than DMPW (Fig. 3f). On the other hand, YI increased gradually ($p < 0.05$) in purified marine PL liposomal dispersions with amino acids added, DMPWA and DLCA (Fig. 3e). Apparently, addition of amino acids to DMPWA and DLCA significantly ($p < 0.05$) decreased the YI development over time as compared to DMPW and DLC. This phenomenon might be due to antioxidative properties of pyrroles or amino acids as mentioned earlier. In addition, hydrophobic pyrroles formation resulting from PE pyrrolisation seemed to be lower (but not statistically significant) over 6 days of incubation in DLCA and DMPWA as compared to DLC and DMPW, respectively (Fig. 3f). In addition, the PE losses also seemed to be lower (but not statistically significant) in DLCA and DMPWA than in DLC and DMPW after 6 days of incubation (Fig. 3h). This phenomenon might be ascribed to occurrence of PE pyrrolisation and amino acids pyrrolisation that were competing with one another and thus reduced the losses of PE in DLCA and DMPWA. However, more replications are needed in future to confirm this observation. As far as the pyrrolisation was concerned, the similar hydrophilic pyrroles formation rate was observed in both DMPWA and DLCA. This might be due to the addition of same amount of amino acids to both

liposomal dispersions, which indicate that pyrrolisation might be depending on the available primary amine groups presents in the reaction mixture. As also shown in Fig. 3f and g, the content of both hydrophobic and hydrophilic pyrroles in liposomal dispersions prepared from purified marine PL seemed to be slightly lower (but not statistically different) on 6 days as compared to 4 days of incubation. This is likely to be due to the consumption of pyrroles as antioxidant by lipid oxidation mechanisms as mentioned earlier.

4.2. Investigation of lipid oxidation

4.2.1. Measurement of *n*-3 derived volatiles in PE and PC liposomal dispersions

Lipid oxidation in DPE and DPC with and without amino acids was investigated through measurement of *n*-3 derived volatiles oxidation products, namely propanol, 2-ethylfuran, 1-penten-3-one, (*E*)-2-hexenal, (*Z*)-4-heptenal, (*E,E*)-2,4-heptadienal and (*E,Z*)-2,6-nonadienal over 6 days of incubation at 60 °C. As shown in Fig. 4a, *n*-3 derived volatiles increased ($p < 0.05$) appreciably in both DPC and DPE after 2 days of incubation. These *n*-3 derived volatiles remained constant or showed no significant increase ($p > 0.05$) in DPC thereafter whereas they slightly decreased ($p < 0.05$) in DPE from 2 to 6 days of incubation. On the other hand, *n*-3 derived volatiles formation remained constant or showed no significant increase ($p > 0.05$) in both DPCA and DPEA over time. The lipid oxidation behaviour in DPE and DPC with and without addition of amino acids is in accordance with our data of the YI development which indicate that occurrence of lipid oxidation is in parallel with browning developed as exemplified by YI development. The lower lipid oxidation in DPCA and DPEA might be attributed to the antioxidative properties of pyrroles or amino acids as mentioned earlier. In addition, DPEA was significantly ($p < 0.05$) less oxidised than DPCA and this phenomenon might be attributed to formation of two types of pyrroles in DPEA and thus an increased total content of antioxidant, which subsequently decreased lipid oxidation. In general, the increment of *n*-3 derived volatile or lipid oxidation was in the order: DPC > DPE > DPCA > DPEA. Secondary aldehydes with carbon chain length six and seven

or volatiles such as (*E,E*)-2,4-heptadienal, (*E,Z*)-2,6-decadienal, etc. are actively involved in non-enzymatic browning reactions after their conversion to tertiary lipid oxidation products under appropriate condition (Lu et al., 2012a; Pokorny & Sakurai, 2002; Thanonkaew et al., 2006). Thus, in order to investigate the changes in volatile profile resulting from non-enzymatic browning reactions, the development behaviour of (*E,Z*)-2,6-nonadienal and (*E,E*)-2,4-heptadienal were investigated. However, development pattern of volatile (*E,E*)-2,4-heptadienal was almost similar to that of *n*-3 volatiles and thus only data of (*E,Z*)-2,6-nonadienal was shown in Fig. 4b. (*E,Z*)-2,6-nonadienal was found to decrease ($p < 0.05$) over time and disappeared in DPCA and DPEA, respectively after addition of amino acids. In addition, this volatile increased slightly (but not statistically significant) in DPE and DPC. Furthermore, (*E,E*)-2,4-heptadienal was also found to decrease over time in DPE and showed a much slower increment in both DPCA and DPEA than DPC (data not shown). The decreases in both (*E,Z*)-2,6-nonadienal and (*E,E*)-2,4-heptadienal could explain the occurrence of non-enzymatic browning reactions in liposomal dispersions containing amine group either from PE or amino acids.

4.2.2. Measurement of *n*-3 derived volatiles in purified marine PL liposomal dispersions

Analogous to DPE and DPC, addition of amino acids significantly ($p < 0.05$) decreased the lipid oxidation in LCA and MPLA liposomal dispersion (Fig. 4c). Lipid oxidation was in order: DLC and DMPW > DMPWA > DLCA. The better oxidative stability in both purified marine PL liposomal dispersions with amino acids added might be attributed to antioxidative properties of pyrroles or amino acids as mentioned earlier. This observation further confirmed our hypotheses that the presence of amino acids may affect the oxidative stability of marine PL liposomal dispersions. In terms of volatile profiles, (*E,Z*)-2,6-nonadienal was not present in purified marine PL liposomal dispersions (DLCA and DMPWA) after addition of amino acids (Fig. 4d). Furthermore, (*E,E*)-2,4-heptadienal decreased over time in both DLCA and DMPWA (data not shown). A plausible explanation for this observation is involvement of these secondary volatiles in non-enzymatic browning reactions.

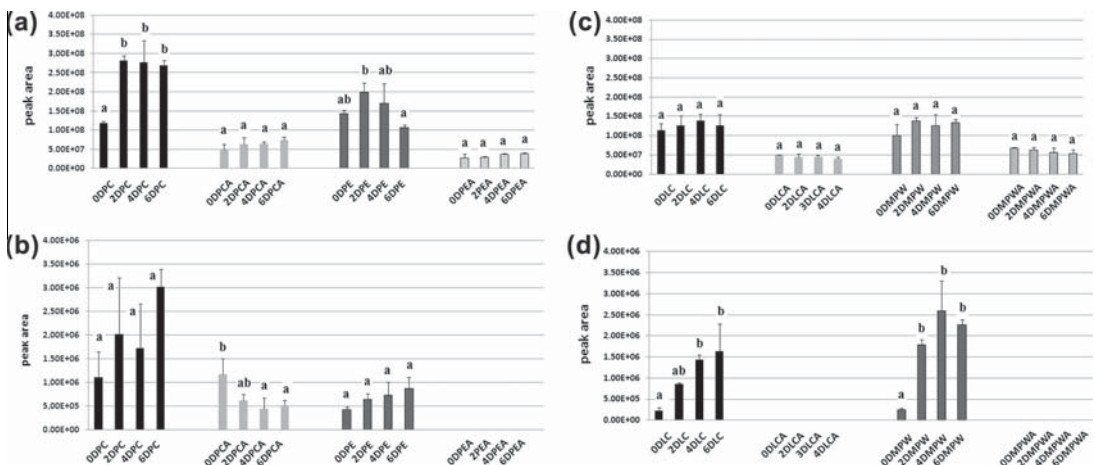


Fig. 4. Measurement of total *n*-3 derived volatiles, which include propanol, 2-ethylfuran, 1-penten-3-one, (*E*)-2-hexenal, (*Z*)-4-heptenal, (*E,E*)-2,4-heptadienal and (*E,Z*)-2,6-nonadienal (a and b); and (*E,Z*)-2,6-nonadienal (c and d) in liposomal dispersions on 0, 2, 4 and 6 days incubations at 60 °C. DPE and DPC are dispersions prepared from authentic standards phosphatidylcholine and phosphatidylethanolamine; DLC and DMPW are dispersions prepared from purified marine PL (LC and MPW); DPEA, DPCA, DLCA and DMPWA are dispersions added with amino acids, namely leucine, methionine and lysine. Values are means \pm standard deviation ($n = 3$).

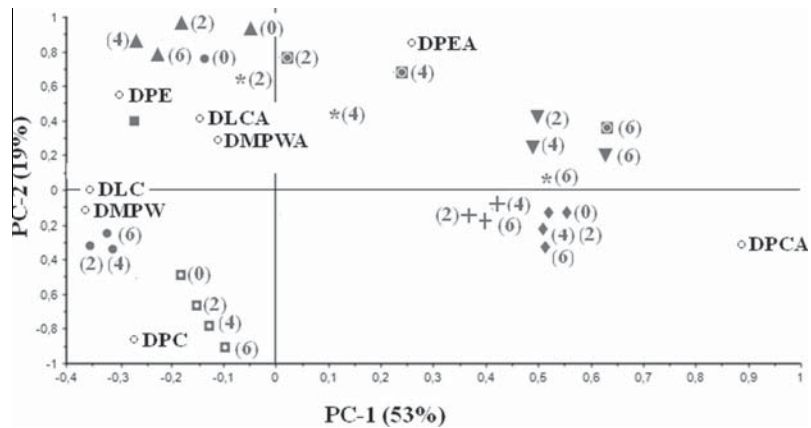


Fig. 5. Bi-plot of principle component analysis for both lipid oxidation and non-enzymatic browning reactions in PL liposomal dispersions incubated at 60 °C for 0, 2, 4 and 6 days: (●) Yellowness index; (□) Total *n*-3 volatiles; (■) PE losses; (▲) hydrophobic pyrroles; (▼) hydrophilic pyrroles; (■) 2-methyl-2-butanal; (+) 2-methyl-2-pentanal; (+) dimethylsulphide; (◆) 3-methylbutanal; (♦) 3-methylbutanal; (0), (2), (4), (6) incubation days; DPE and DPC are dispersions prepared from authentic standards phosphatidylcholine and phosphatidylethanolamine; DLC and DMPW are dispersions prepared from purified marine PL (LC and MPW); DPEA, DPCA, DLCA and DMPWA are dispersions added with amino acids, namely leucine, methionine and lysine.

4.3. Multivariate data analysis

In order to get an overview of different stability patterns of liposomal dispersions, a principle component analysis was made for all liposomal dispersions prepared (as shown in Fig. 5). The purpose of this analysis was to study the correlation between lipid oxidation and non-enzymatic browning reactions. Liposomal dispersions containing no amino acids (DPE, DPC, DLC and DMPW) are located to the left in the plot and liposomal dispersions move to the right in the plot with increasing Strecker derived volatiles from amino acids degradation (Fig. 5). Thus, DPCA which is located further to the right in the plot had the highest content of Strecker derived volatiles as it is located near to Strecker derived volatiles such as 3-methylbutanal, dimethylsulphide, 2-methyl-2-butanal and 2-methyl-2-pentanal. In addition, dispersions containing no amino acids are located nearer to the *n*-3 derived volatiles and yellowness index (YI) than dispersions containing amino acids indicating that a higher degree of lipid oxidation and browning occurred in dispersions without amino acids. The close proximity between *n*-3 derived volatiles and YI indicating a clear positive correlation between lipid oxidation and browning development as also exemplified by DPC, which showed the highest degree of lipid oxidation and browning. Furthermore, DPC is located far away from variables of pyrrole indicating that browning in DPC was not due to the amine group pyrrolisation as also confirmed by no pyrroles detection in DPC dispersion. On the other hand, DPCA with the lowest level of browning and lipid oxidation (located far away from both variables of *n*-3 derived volatiles and YI, but close to variable of hydrophilic pyrrole) had the highest level of hydrophilic pyrroles.

In addition, a close proximity between hydrophobic pyrroles and PE losses on the upper left in the plot indicated a positive correlation between PE losses and formation of hydrophobic pyrroles in liposomal dispersions containing PE, namely DPE, DLC, DMPW, DLCA and DMPWA. On the other hand, liposomal dispersions containing amino acids (DPEA and DPCA) are located near to the variable of hydrophilic pyrrole on the right in the plot indicating that a higher content of hydrophilic pyrrole was found in these liposomal dispersions. In general, liposomal dispersions containing amine group either from PE or amino acids (DPE, DPEA, DPCA, DLCA and DMPWA) were located nearer to the pyrrole variables and farther away from the variable of *n*-3 derived volatiles. All these

observations indicated a negative correlation between lipid oxidation and pyrroles or amino acids content. To summarize, the obtained results from multivariate data analysis supported our hypothesis that the presence of amino acids and pyrrole may decrease the lipid oxidation in liposomal dispersions prepared from PC, PE and purified marine PL. In addition, the presence of amino acids and pyrrole also partly account for non-enzymatic browning development, especially the SD reaction.

5. Conclusion

Oxidative stability of liposomal dispersions was greatly influenced by the presence of amine group from PE or amino acids. The presence of PE and amino acids most likely accounted for the occurrence of non-enzymatic browning reactions, SD and pyrrolisation in the liposomal dispersions. The occurrence of SD was observed from the presence of Strecker derived volatiles, namely 3-methylbutanal, dimethylsulphide, 2-methyl-2-butanal and 2-methyl-2-pentanal as degradation products from amino acids in the liposomal dispersions; whereas the occurrence of pyrrolisation was observed from the presence of hydrophobic pyrroles (PE pyrrolisation) and hydrophilic pyrroles (amino acids pyrrolisation). In addition, pyrrolisation and oxypolymerisation were responsible for YI development in liposomal dispersions containing amine group, whereas only oxypolymerisation was responsible for YI development in liposomal dispersion containing no primary amine group such as PC liposomal dispersion. In general, the presence of PE and amino acids lowered the YI development and decreased lipid oxidation. This phenomenon might be attributed to the antioxidative properties of pyrroles formed in non-enzymatic browning reactions or to the antioxidative properties of added amino acids.

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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.2013.03.063>.

References

- Applegate, K. R., & Glomset, J. A. (1986). Computer-based modeling of the conformation and packing properties of docosahexaenoic acid. *Journal of Lipid Research*, 27, 658–680.
- Chen, H. M., Muramoto, K., Yamauchi, F., & Nokihara, K. (1996). Antioxidant activity of designed peptides base on the antioxidant peptide isolated from digests of a soybean protein. *Journal of Agricultural and Food Chemistry*, 44, 2619–2623.
- Cho, S. Y., Joo, D. S., Choi, H. G., Nara, E., & Miyashita, K. (2001). Oxidative stability of lipids from squid tissues. *Fisheries Science*, 67, 738–743.
- Elias, R. J., McClements, D. J., & Decker, E. A. (2005). Antioxidant activity of cysteine, tryptophan and methionine residues in continuous phase β -lactoglobulin in oil in water emulsions. *Journal of Agricultural and Food Chemistry*, 53, 10248–10253.
- Francis, F. J., & Clydesdale, F. H. (1975). *Food colorimetry: Theory and application*. Westport, CT: AVI Publishing.
- Guo, H., Kouzuma, Y., & Yonekura, M. (2009). Structures and properties of antioxidative peptides derive from royal jelly protein. *Food Chemistry*, 113, 238–245.
- Hidalgo, F. J., Mercedes leoan, M., & Zamora, R. (2006). Antioxidative activity of amino phospholipids and phospholipid/amino acid mixtures in edible oils as determined by the Rancimat method. *Journal of Agricultural and Food Chemistry*, 54, 5461–5467.
- Hidalgo, F. J., Mercedes leoan, M., Nogales, F., & Zamora, R. (2007). Effect of tocopherols in the antioxidative activity of oxidized lipid–amine reaction products. *Journal of Agricultural and Food Chemistry*, 55, 4436–4442.
- Hidalgo, F. J., Nogales, F., & Zamora, R. (2003). Effect of the pyrrole polymerization mechanism on the antioxidative activity of nonenzymatic browning reactions. *Journal of Agricultural and Food Chemistry*, 51, 5703–5708.
- Hidalgo, F. J., Nogales, F., & Zamora, R. (2004). Determination of pyrolyzed phospholipids in oxidized phospholipid vesicles and lipoproteins. *Analytical Biochemistry*, 334, 155–163.
- Hidalgo, F. J., Nogales, F., & Zamora, R. (2005a). Changes produced in the antioxidative activity of phospholipids as a consequence of their oxidation. *Journal of Agricultural and Food Chemistry*, 53, 659–662.
- Hidalgo, F. J., Nogales, F., & Zamora, R. (2005b). Nonenzymatic browning, fluorescence development, and formation of pyrrole derivatives in phosphatidylethanolamine/ribose/lysine model systems. *Journal of Food Science*, 70, 387–391.
- Hidalgo, F. J., & Zamora, R. (1993). Fluorescent pyrrole products from carbonyl–amine reactions. *Journal of Biological Chemistry*, 268, 16190–16197.
- Hidalgo, F. J., & Zamora, R. (2004). Strecker-type degradation produced by the lipid oxidation products 4,5-epoxy-2-alkenals. *Journal of Agricultural and Food Chemistry*, 52, 7126–7131.
- Khayat, A., & Schwall, D. (1983). Lipid oxidation in seafood. *Food Technology*, 37, 130–1400.
- Kim, H. Y., & Salem, N. (1990). Separation of lipid classes by solid phase extraction. *Journal of Lipid Research*, 31, 2285–2289.
- Le Grandois, J., Marchioni, E., Zhao, M. J., Giuffrida, F., Ennahar, S., & Bindler, F. (2009). Investigation of natural phosphatidylcholine sources: Separation and identification by liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS2) of molecular species. *Journal of Agricultural and Food Chemistry*, 57, 6014–6020.
- Levine, R. L., Mosoni, L., Berlett, B. S., & Stadman, E. R. (1996). Methionine residues as endogenous antioxidant in proteins. *Proceedings of the National Academy of Sciences*, 93, 15036–15040.
- Lu, F. S. H., Nielsen, N. S., Timm-Heinrich, M., & Jacobsen, C. (2011). Oxidative stability of marine phospholipids in the liposomal form and their applications. *Lipids*, 46, 3–23.
- Lu, F. S. H., Nielsen, N. S., Baron, C., & Jacobsen, C. (2012a). Oxidative degradation and non-enzymatic browning between oxidized lipids and primary amine groups in different marine PL emulsions. *Food Chemistry*, 135, 2887–2896.
- Lu, F. S. H., Nielsen, N. S., Baron, C., Jensen, L. H. S., & Jacobsen, C. (2012b). Physico-chemical properties of marine phospholipid emulsions. *Journal of the American Oil Chemists' Society*, 89, 2011–2024.
- Miyashita, K., Nara, E., & Ota, T. (1994). Comparative-study on the oxidative stability of phosphatidylcholines from salmon egg and soybean in an aqueous-solution. *Biosciences, Biotechnology, and Biochemistry*, 58, 1772–1775.
- Moriya, H., Kunimino, T., Hosokawa, M., Fukunaga, K., Nishiyama, T., & Miyashita, K. (2007). Oxidative stability of salmon and herring roe lipids and their dietary effect on plasma cholesterol levels of rats. *Fisheries Science*, 73, 668–674.
- Peng, J. L., Larondelle, Y., Pham, D., Ackman, R. G., & Rollin, X. (2003). Polyunsaturated fatty acid profiles of whole body phospholipids and triacylglycerols in anadromous and landlocked Atlantic salmon (*Salmo salar* L.) fry. *Comparative Biochemistry and Physiology Part B*, 134, 335–348.
- Pokorny, J., & Sakurai, H. (2002). Role of oxidized lipids in nonenzymatic browning reactions. *International Congress Series*, 1245, 373–374.
- Thanonkaew, A., Benjakul, S., Visessanguan, W., & Decker, E. A. (2006). Development of yellow pigmentation in squid (*Loligo peali*) as a result of lipid oxidation. *Journal of Agricultural and Food Chemistry*, 54, 956–962.
- Thanonkaew, A., Benjakul, S., Visessanguan, W., & Decker, E. A. (2007). Yellow discoloration of the liposome system of cuttlefish (*Sepia pharaonis*) as influenced by lipid oxidation. *Food Chemistry*, 102, 219–224.
- Uematsu, T., Parkanyiova, L., Endo, T., Matsuyama, C., Yano, T., Mitsuyoshi, M., Sakurai, H., & Pokorny, J. (2002). Effect of the unsaturation degree on browning reactions of peanut oil and other edible oils with proteins under storage and frying conditions. *International Congress Series*, 1245, 445–446.
- Ventanas, S., Estevez, M., & Delgado, C. L. (2007). Phospholipid oxidation, non-enzymatic browning development and volatile compounds generation in model systems containing liposomes from porcine *Longissimus dorsi* and selected amino acids. *European Food Research and Technology*, 225, 665–675.
- Wijendran, V., Huang, M. C., Diau, G. Y., Boehm, G., Nathanielsz, P. W., & Brenna, J. T. (2002). Efficacy of dietary arachidonic acid provided as triglyceride or phospholipid as substrates for brain arachidonic acid accretion in baboon neonates. *Pediatric Research*, 51, 265–272.
- Zamora, R., Alaiz, M., & Hidalgo, F. J. (2000). Contribution of pyrrole formation and polymerization to the nonenzymatic browning produced by amino-carbonyl reactions. *Journal of Agricultural and Food Chemistry*, 48, 3152–3158.
- Zamora, R., Gallardo, E., & Hidalgo, F. (2007). Strecker degradation of phenylalanine initiated by 2,4-decadienal or methyl 13-oxooctadeca-9,11-dienoate in model systems. *Journal of Agricultural and Food Chemistry*, 55, 1308–1314.
- Zamora, R., & Hidalgo, F. J. (2005). Coordinate contribution of lipid oxidation and Maillard reaction to the nonenzymatic food browning. *Critical Reviews in Food Science and Nutrition*, 45, 49–59.
- Zamora, R., & Hidalgo, F. J. (2011). The Maillard reaction and lipid oxidation. *Lipid Technology*, 23, 59–62.
- Zamora, R., Nogales, F., & Hidalgo, F. J. (2005). Phospholipid oxidation and nonenzymatic browning development in phosphatidylethanolamine/ribose/lysine model systems. *European Food Research and Technology*, 220, 459–465.
- Zamora, R., Rios, J. J., & Hidalgo, F. J. (1994). Formation of volatile pyrrole products from epoxyalkenals/protein reactions. *Journal of Agricultural and Food Chemistry*, 66, 543–546.

PAPER VI

Lu, F. S. H., Thomsen, B. R., Hyldig, G., Green-Petersen, D. M. B., Nielsen, N. S., Baron, C. P., Jacobsen, C.

Oxidative stability and sensory attributes of fermented milk product fortified with a neat or pre-emulsified mixture of fish oil and marine phospholipids.
Journal of the American Oil Chemists' Society 2013 (Resubmitted after revision)

1 **Oxidative stability and sensory attributes of fermented milk product**
2 **fortified with fish oil and marine phospholipids**

3 Lu, F. S. H., Thomsen, B. R., Hyldig, G., Green-Petersen, D. M. B., Nielsen, N. S., Baron, C.
4 P., Jacobsen, C.*

5 Division of Industrial Food Research, Technical University of Denmark, Søtofts Plads,
6 Building 221, 2800 Kgs. Lyngby, Denmark.

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21 Running title: Oxidative stability and sensory attributes of marine lipids fortified fermented
22 milk product.

23 Keywords: Marine phospholipids, fish oil, oxidative stability, emulsion, fermented milk
24 product, sensory evaluation, fishy, rancid, secondary volatiles.

25
26
27 **Corresponding author.**

28 Tel: +4545252559; fax: +4545884774

29 Email: chja@food.dtu.dk. Charlotte Jacobsen

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1 **ABSTRACT:**

2 Marine phospholipid (PL) is a potential ingredient for food fortification due to its numerous
3 advantages. The main objective of this study was to investigate if a fermented milk product
4 fortified with a mixture of marine PL and fish oil had better oxidative stability than a
5 fermented milk product fortified with fish oil alone. Fortification of fermented milk product
6 with marine PL was performed by incorporating 1 % w/w lipids, either in the form of neat oil
7 or in the form of pre-emulsion. Lipid oxidation was investigated in the neat emulsions and
8 fortified products by the measurements of primary, secondary volatile oxidation products and
9 tocopherol content upon 32 days storage at 2 °C and 28 days storage at 5 °C, respectively.
10 Analyses of particle size distribution, viscosity and microbial growth were also performed. In
11 addition, sensory attributes such as sour, fishy and rancid flavour/odour were evaluated in
12 fortified products by a trained panel. The obtained results showed that incorporation of a
13 mixture of marine PL and fish oil into fermented milk products decreased the oxidative
14 stability and sensory quality of fortified products. The pH depending behaviour of iron
15 seemed to be the main factor that influenced the lipid oxidation in marine PL emulsion and
16 fermented milk system. In addition, both oxidative stability and sensory acceptability of
17 fortified products varied depending on the quality of marine PL used for fortification.

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2 **INTRODUCTION:**

3 Marine phospholipids (PL) have received much attention recently as they have been
4 suggested to provide more advantages than marine triglycerides (TAG) available from fish
5 oil. These advantages include: i) higher content of physiologically important n-3
6 polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA) and
7 docosahexaenoic acid (DHA) [1]; ii) better bioavailability of EPA and DHA [2]; iii) better
8 resistance towards oxidation due to the antioxidative properties of PL [3, 4] and iv) a broader
9 spectrum of health benefits including those from n-3 PUFA, and their polar head groups or a
10 combination of the two in the same molecule [5]. The health benefits of n-3 PUFA include
11 their ability to reduce coronary heart diseases, inflammation, autoimmune diseases,
12 hypertension, cancer, diabetes, mental illness and neurological diseases, as well as
13 improvement for brain and eye functions in infants as reviewed by Narayan and colleagues
14 [6]. The health benefits of marine PL in general, as reviewed in our previous publication [7]
15 include their therapeutic value for metabolic syndrome, hyperlipidemia, non-alcoholic fatty
16 liver disease, attention deficit/hyperactivity deficit disorder (AD/HD), premenstrual
17 syndrome (PMS) and anti-inflammatory effect as demonstrated in several studies of krill PL.

18 Due to numerous advantages of marine PL, there is a growing awareness of their
19 potential use as ingredient for food fortification. In addition, marine PL are potentially
20 interesting natural surfactants to prepare food emulsions. Thus, marine PL emulsions can be
21 used as effective carrier of n-3 PUFA due to the fact that they can be easily incorporated into
22 emulsified food. Currently, there is a wide range of n-3 PUFA preparations in the form of
23 TAG oil and powder that are available for food fortification in the market [8]. In contrast, the
24 use of marine PL as food ingredient is just starting to be explored by the food industry. For
25 instance, several krill oil producing companies have obtained Generally Recognized as Safe

1 (GRAS) status for krill derived lecithin or krill oils, which are rich in PL [9-11]. The current
2 limited application of marine PL in food industry can be attributed to several reasons such as
3 lack of knowledge especially related to the stability of marine PL in food systems and marine
4 PL quality issues.

5 There are many studies on fish oil fortified products available in literature, but to the
6 best of our knowledge, there is scarce information on food fortification with marine PL
7 except a few recent studies on krill oil food fortification. Amongst these is fortification of
8 surimi and egg products with krill oil. The quality of these fortified products was compared to
9 that of flaxseed, algae, menhaden and a blend of these oils [12-14]. These studies reported
10 that food fortification with krill oil increased the n-3 PUFA content and susceptibility of the
11 products towards lipid oxidation due to the high content of n-3 PUFA in krill oil. Despite the
12 occurrence of lipid oxidation, the fortified products were still acceptable with respect to their
13 sensory properties. In addition, several studies on oxidative stability of marine PL liposomes
14 under gastrointestinal condition have been reported [15-17], and studies on physico-chemical
15 properties, oxidative stability and non-enzymatic browning reactions in marine PL emulsion
16 were recently reported in our previous publications [18-20]. The main objective of this study
17 was to investigate if a fermented milk product fortified with a mixture of fish oil and marine
18 PL had better oxidative stability than a fermented milk product fortified with fish oil in TAG
19 form, and this included the investigation of how the quality of different marine PL sources
20 affected the oxidative stability and sensory acceptability of fortified fermented milk product.
21 The secondary objective was to investigate the feasibility of using marine PL either in the
22 neat or pre-emulsified form for fermented milk product fortification. According to a study of
23 Let and colleagues [21], the use of marine oils in stabilized pre-emulsified form for food
24 fortification could protect n-3 PUFA from oxidation during the processing. It has been
25 proposed that the interaction between n-3 PUFA in marine oil and potential pro-oxidative

1 compounds in food systems at the interface membrane could be reduced when n-3 PUFA
2 were incorporated as pre-emulsion, thereby preventing oxidative degradation. The oxidative
3 stability of the fermented milk products fortified with marine PL emulsions was compared
4 with that of the parent marine PL emulsions.

5

6 **MATERIALS**

7 Two different marine phospholipids (MGK and MPN) were obtained from Polaris (Pleuven,
8 France) and Triple Nine (Esbjerg, Denmark), respectively. Compositions of both marine PL
9 are shown in Table 1. Fish oil (Maritex 43-01) was supplied by Maritex (Subsidiary of TINE
10 BA, Sortland, Norway). This fish oil had low initial PV (0.16 meq/kg) and contained 240.4
11 mg/kg α -tocopherol, 99.3 mg/kg γ -tocopherol and 37.9 mg/kg δ -tocopherol. Fermented milk
12 product of 1.5 % fat content (A38, Arla, Slagelse, Denmark) was purchased locally. All
13 solvents were of HPLC grade (Lab-Scan, Dublin, Ireland).

14

15 **METHODS**

16 **Preparation of marine PL emulsion:**

17 Different formulations of marine PL emulsion (300 mL for each formulation) were prepared
18 from a mixture of PL and fish oil (Table 2a). Emulsions were prepared in two steps; pre-
19 emulsification and homogenization. In the pre-emulsification step, a combination of fish oil
20 and marine PL were added to 10mM acetate-imidazole (pH 7) buffer solution over 1 min
21 under vigorous mixing (19,000 rpm) with an Ultra-Turrax (Ystral, Ballrechten-Dottingen,
22 Germany) followed by 2 min of mixing at the same speed. All pre-emulsions were
23 subsequently homogenized in a Panda high pressure table homogenizer (GEA Niro Soavi
24 SPA, Parma, Italy) using a pressure of 800 bar and 80 bar for the first and second stages,
25 respectively. After homogenization, 1 mL of sodium azide (10%) was added to each

1 emulsion (220 g) to inhibit microbial growth. Emulsions (220 g for each formulation) were
2 stored in 250 mL blue cap bottles at 2°C in darkness for 32 days. Samples were taken on day
3 0, 4, 8, 16 and 32, flushed with nitrogen and stored at - 40 °C until further analysis. Samples
4 were analyzed for their oxidative stability, which included the measurement of peroxide
5 value (PV) and measurement of secondary volatile oxidation products by Solid Phase
6 Microextraction (SPME) GC-MS. Emulsions were also prepared with the same method as
7 above by using water instead of buffer and without addition of antimicrobial solution for the
8 use of fermented milk product fortification.

9

10 **Fortification and storage studies**

11 Fortification of fermented milk product with neat marine PL or fish oil or a mixture of them
12 in emulsion form was carried out according to the experimental design as shown in Table 2b.
13 Firstly, the weighed fermented milk product was added to a Stephan-mixer (Stephan,
14 Hameln, Germany) and stirred under cold and vacuum condition at 1200rpm. Then, fish oil
15 or marine PL or a mixture of both in different forms was added slowly to the mixture and
16 stirring was continued for another 1 min. In order to prepare fermented milk product fortified
17 with 1% w/w marine PL, 10 g of the 10 % emulsion or 2 g of 50% emulsion was added to
18 100 g of fermented milk product. The fortified products were stored for 4 weeks at 5°C and
19 sampling was done at 5 time intervals (day 0, 7, 14, 21 and 28) for measurement of peroxide
20 value (PV), secondary volatile oxidation products, and tocopherol content. In contrast to
21 chemical analysis, the sensory evaluation was not done at day 28, but only at day 0, 7, 14 and
22 21 on the selected fortified products. Fermented milk product fortified with neat MPN and
23 MGK were excluded from sensory evaluation as these products showed strong off flavour
24 even before the storage. All samples were flushed with nitrogen gas and stored at -40 °C prior
25 to the sensory evaluation and chemical analysis.

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Determination of particle size distribution

Droplet sizes were determined in the different emulsions using laser diffraction with a Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire, UK). Approximately eight drops of emulsion were suspended directly in recirculating water (3000 rpm), reaching an obscuration at 15–18%. The refractive indices of sunflower oil (1.469) and water (1.330) were used as particle and dispersant, respectively. The analysis was performed in triplicate.

Determination of viscosity

Viscosity of the emulsion samples was measured in triplicate using a Stress Tech (Rheologica Instruments, AB, Lund, Sweden) with the program Stress Tech 3.8 at 5 °C from shear rate 0 to 17.49 rad/s in 300s and back to 0 in 300s.

Measurement of lipid oxidation

a) Determination of peroxide value

Lipids were extracted from the emulsions or fermented milk products according to the Bligh and Dyer method using a reduced amount of the chloroform/methanol (1:1 w/w) solvent [22]. Two extractions were made from each sample and the measurement was performed in duplicate. PV was measured by the colorimetric ferric-thiocyanate method at 500 nm using a spectrophotometer (Shimadzu UV-160A, UV-Vis, Struers Chem A/S, DK) as described by International IDF [23] and Shantha & Decker [24].

b) Determination of tocopherol content

Lipid extracts from Bligh & Dyer were weighed (1-2 g) and evaporated under nitrogen and re-dissolved in heptane prior to tocopherol analysis by HPLC (Agilent 1100 series, Agilent

1 Technologies, Palo Alto, CA, USA). A Water Spherisorb (R) silica column (4.6 x 150 mm,
2 i.d. = 3 μ m) was used for tocopherol analysis. The mobile phase consisted of heptane and iso-
3 propanol (100:0.4, respectively) and was introduced at a flow rate of 1 mL/min. Tocopherols
4 were detected with a fluorescence (FLD) detector at 290 nm as excitation wavelength and at
5 330 nm as emission wavelength according to the AOCS Official Method Ce 8-89 [25]. Two
6 extractions were made from each sample and the measurement was performed in duplicate
7 and quantified by authentic standards.

8

9 ***c) Headspace analysis using solid phase microextraction (SPME) GC-MS***

10 Approximately 1 g of emulsion, together with 30 mg of internal standard (10 μ g/g of 4-
11 methyl-1-pentanol in rapeseed oil) was mixed on a whirly mixer for 30 seconds in a 10 mL
12 vial. The sample was equilibrated for 3 min at a temperature of 60°C, followed by extraction
13 for 45 min at the same temperature while agitating the sample at 500 rpm. Headspace
14 volatiles were adsorbed to a 50/30 μ m CAR/PDMS SPME fiber (Supelco, Bellafonte, PA,
15 USA) installed on a CTC Combi Pal (CTC Analytics, Waldbronn, Germany). Volatiles were
16 desorbed in the injection port of gas chromatograph (HP 6890 Series, Hewlett Packard, Palo
17 Alto, CA, USA; Column: DB-1701, 30 m x 0.25 mm x 1.0 μ m; J&W Scientific, CA, USA)
18 for 60 seconds at 220°C. The oven program had an initial temperature of 35°C for 3 min,
19 with increment of 3.0°C/min to 140°C, then increment of 5.0°C/min to 170°C and increment
20 of 10.0°C/min to 240°C, where the temperature was held for 8 min. The individual
21 compounds were analyzed by mass-spectrometry (HP 5973 inert mass-selective detector,
22 Agilent Technologies, USA; Electron ionization mode, 70eV, mass to charge ratio scan
23 between 30 and 250). In order to investigate lipid oxidation in marine PL emulsions, the
24 following secondary volatiles were selected for quantification: pentanal, hexanal octanal,
25 nonanal, *E*-2-hexenal, 1-penten-3-one, *Z*-4-heptenal, and *E,E*-2,4-heptadienal. However, only

1 one volatile, which showed appreciable changes over time was chosen for comparison and
2 discussion. Calibration curves were made by dissolving the different volatile standards in
3 rapeseed oil followed by dilution to obtain different concentrations (0.1-10µg/g) prior to
4 addition of them into emulsions. Due to the different retention capacity of volatiles in
5 emulsions with different formulations/matrix, two set of calibration curves were prepared; a
6 matrix of emulsion containing 10% marine lipids and a matrix of emulsion containing 50%
7 marine lipids. In this study calibration curves were parallel shifted in order to obtain positive
8 values. The given values (in ng/g) of the volatiles are thus not accurate values and should
9 therefore not be used for comparison to other studies. Measurements were made in triplicates
10 on each emulsion.

11

12 *d) Headspace analysis using dynamic headspace (DHS) GC-MS analysis*

13 Volatiles from 4 ml of fortified fermented milk product were collected by purging the sample
14 with nitrogen (150 ml/min) for 30 min at 45 °C, using 4-methyl-1-pentanol as the internal
15 standard, and trapped on Tenax GR tubes (Perkin-Elmer, CN, USA) packed with 225 mg
16 Tenax GR (60-80 mesh, Varian, Middelburg, Netherlands). The volatiles were desorbed at
17 200 °C in an automatic thermal desorber (ATD-400, Perkin-Elmer, Norwalk, CT) and
18 cryofocused on a Tenax GR cold trap. Volatiles were separated by gas chromatography (HP
19 5890 IIA, Hewlett-Packard, Palo Alto, CA) as described by Timm-Heinrich and colleagues
20 [26] and analyzed by mass spectrometry (HP 5972 mass selective detector). The oven
21 temperature program was: 45 °C held for 5 min, 1.5 °C /min to 55 °C, 2.5 °C /min to 90 °C,
22 12 °C /min to 220 °C and finally held at 220 °C for 4 min. One of the n-3 PUFA derived
23 volatiles identified by MS-library searches (Wiley 138 K, John Wiley and Sons, Hewlett-
24 Packard) and authentic external standard, namely 1-penten-3-ol was chosen for
25 quantification. Calibration curve was made by dissolving the standard of this volatile in

1 rapeseed oil followed by dilution to obtain different concentrations (0.001-0.05mg/g) prior to
2 addition of 1 µl of these standards into plain fermented milk product sample.

3

4 **Microbiology**

5 Microbial growth in fermented milk product stored for 21 days was investigated through
6 preparation of different agar plates such as nitrate actidion polymyxin (NAP) agar (ATP
7 265430, Difco, MA, USA), Oxytetracycline Glucose Yeast Extract agar (OGYE, M0545,
8 Oxoid, Hampshire, UK) with selective supplement, Long & Hammers' agar [27] and
9 physiological saline (0.85% NaCl and 0.1 % Bacto-Peptide). The prepared agar plates and
10 solutions were incubated accordingly to investigate the content of total bacteria,
11 Lactobacillus, general lactic acid bacteria and yeast. Single measurement was made for each
12 sample and the plates were visually inspected.

13

14 **Sensory evaluation**

15

16 Eleven trained panelists were recruited to evaluate the marine PL fortified products using
17 objective descriptive sensory profiling. The panel was recruited and trained in descriptive
18 sensory analysis according to standards [28, 29]. The panel had undergone three training
19 sessions for the following attributes: fishy, rancid and sour both for aroma (orthonasal) and
20 for flavour (retronasal). In this two to three hours training sessions, the panel was trained by
21 using the similar products as used in the present study. The fortified products were served at
22 5 °C in order to mimic realistic consumer behaviour. In addition, the panel was served with
23 diluted apple juice and crisp bread to rinse their mouths between the samples. Samples were
24 served in plastic cups with a lid (20 mL). The samples were blinded using three-digit codes
25 and served in randomized order to minimize carryover effects. The panel was instructed to
26 shake the samples before evaluating them in order to ensure their homogeneity. All samples

1 were evaluated in duplicates. All sensory attributes were rated on an unstructured 15 cm line
2 scale with anchor points 1.5 cm from each end. The data were recorded on computers by
3 using the FIZZ program (Biosystems, Couternon, France). In total, 4 sessions of sensory
4 evaluation were performed, six to seven samples were tested in each session. The evaluations
5 were performed in separated booths under normal daylight and at ambient temperatures
6 according to ISO standards [30]. The obtained sensory data were calculated by determining
7 the overall mean scores for intensity.

8

9 **Statistical Analysis**

10 The data of measurements such as PV, secondary volatile oxidation products, tocopherol
11 content and sensory data were subjected to one way and two ways ANOVA analysis and
12 comparison among samples were performed with *Bonferroni* multiple comparison test using
13 a statistical package program Graphpad Prism 4 (Graphpad Software Inc., San Diego, USA).
14 Significant differences were accepted at $p < 0.05$.

15

16 **RESULTS AND DISCUSSION**

17

18 **Chemical composition of marine PL**

19

20 In this study, two commercial marine PL from different sources, namely MPN and MGK
21 were used. MPN was extracted from sprat fish meal whereas MGK was extracted from
22 Antarctic krill *Euphausia Superba* and therefore they had different chemical composition and
23 quality (Table 1). In terms of phospholipids content, MPN had slightly higher PL content
24 than MGK (44% vs 40%). However, the opposite was the case for PC content, as the PC
25 content in MPN was slightly lower than MGK (22% vs 32%). MPN and MGK had almost
26 similar content of phosphatidylethanolamine (PE) and phosphatidylinositol (PI),
27 approximately 4% and 2%, respectively. In addition, MPN also contained 3.5% of

1 sphingomyelin (SPM) and 12.6% of unknown PL, whereas MGK did not contain SPM and
2 had only 2% unknown PL.

3 There were slight differences in EPA and DHA content between the two marine PL
4 preparations. MPN contained a lower amount of EPA but a higher amount of DHA as
5 compared to that of MGK (8.4g/100g EPA and 12.5 g/100g DHA in MPN vs 11.0g/100g
6 EPA and 7.0g/100g DHA in MGK). Therefore, MPN had higher degree of unsaturation than
7 MGK. In terms of antioxidant content, MGK had three times higher α -tocopherol content
8 than MPN. In addition to α -tocopherol, MGK also contained 50 m g/Kg of esterified
9 astaxanthin, whereas MPN contained less than 10 mg/Kg of ethoxyquin (as shown in Table
10 1). Ethoxyquin is a well known antioxidant used in fish meal and therefore it was co-
11 extracted from fish meal during marine PL production.

12 As far as the quality of marine PL was concerned, MGK contained lower levels of
13 transition metal (iron) and initial hydroperoxides than MPN. In addition, MGK was found to
14 be less oxidized than MPN through the measurement of initial n-3 derived volatiles (data not
15 shown). In conclusion, MGK was considered to have better quality than MPN as it contained
16 higher levels of antioxidants and less prooxidants.

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19 **Lipid oxidation in marine PL emulsion**

20
21 Marine PL emulsions were prepared from a mixture of both marine PL preparation and fish
22 oil according to experimental design shown in Table 2a. All emulsions showed PV
23 increments over 32 days storage (as shown in Figure 1a). The 10 % MGK emulsion showed a
24 significantly higher PV increment during storage than that of 50 % MGK emulsion, followed
25 by 10% MPN emulsion and 50% MPN emulsion. The interpretation of oxidative stability of
26 any sample can, however, not solely be based on PV measurements. Low PV in marine PL
27 emulsions may indicate fast decomposition of hydroperoxides into secondary oxidation

1 products [19, 31] and thus measurement of secondary oxidation products must be taken into
2 consideration in order to get a more accurate interpretation of lipid oxidation in marine PL
3 emulsions. In this study, data for secondary volatiles oxidation products showed a similar
4 pattern of increment in marine PL emulsions as observed for PV (as shown for 2,4-
5 heptadienal in Figure 1b). Taken together, the order of lipid oxidation was ranked as follows:
6 10% MGK > 50% MGK > 10% MPN > 50% MPN.

7 MGK emulsions had a larger population of bigger droplets (mean diameter at peak
8 5 μ M) as shown in Figure 1c. In contrast to 10 % MGK emulsion, 10% MPN emulsion had a
9 higher proportion of smaller droplets (mean diameter at peak 0.1 μ M). As also reported in our
10 previous study [18], bigger droplets (mean diameter at peak 5 μ M) might indicate the
11 presence of TAG oil droplets surrounded by PL, whereas the smaller droplets (mean diameter
12 at peak 0.1 μ M) might indicate the presence of PL liposomes as reported in study by Mozafari
13 and colleagues [32]. However, this needs to be investigated further. PL can spontaneously
14 self-assemble and form liposomes in the presence of water. It is therefore most likely that
15 such structures were formed during the homogenization in addition to the formation of
16 emulsified oil droplets [18]. In addition, it is speculated that the presence of a larger
17 population of PL liposomes in MPN emulsion, especially those formed from PL containing
18 PUFA at sn-2 position might provide a more tightly packed molecular conformation. This
19 favourable physical structure of the 10% MPN emulsions could have reduced the attack of
20 free radicals and oxygen toward PUFA in the bilayers of tighter conformation and thereby
21 decreased lipid oxidation in MPN [33].

22 Furthermore, the higher degree of lipid oxidation in both 10% and 50% MGK
23 emulsions was confirmed by the higher percentage of α -tocopherol consumption during lipid
24 oxidation in these emulsions. As shown in Figure 1d, the content of α -tocopherol decreased
25 faster in MGK emulsion as compared to that of MPN emulsion (23.6 % of decrement in 10%

1 MPN emulsion vs 100 % decrement in 10 % MGK emulsion and 12.4 % of decrement in
2 50% MPN emulsion vs 53.91 % of decrement in 50% MGK emulsion).

3 In terms of the effect of lipid content in emulsion on lipid oxidation, emulsions
4 prepared from 10 % lipid content was more oxidized than those with 50% for both MGK and
5 MPN. This phenomenon might be attributed to the lower viscosity in 10 % emulsion and
6 thereby increased diffusion of oxygen and prooxidants and subsequently enhanced lipid
7 oxidation [34].

8 9 **Lipid oxidation in fortified products**

10 Incorporation of marine PL either in neat form or in pre-emulsified form significantly ($p <$
11 0.05) increased the PV of the fortified products as compared to plain fermented milk product
12 or product with neat fish oil added during 21 days storage at $5\text{ }^{\circ}\text{C}$ (Figure 2a). Due to the
13 different contents of marine PL in fermented milk products prepared with neat marine PL oils
14 and pre-emulsified oils, comparison of lipid oxidation was categorized into 2 groups; a group
15 comprising fermented milk products fortified with neat oil (MGK, MPN and fish oil); a group
16 comprising fermented milk products fortified with emulsion (10% or 50% emulsions
17 prepared from MGK or MPN, respectively).

18
19 Fermented milk product fortified with neat MPN showed a larger PV increment ($p <$
20 0.05) than fermented milk product fortified with neat MGK followed by neat fish oil. In
21 contrast to PV measurement, volatiles data seemed to show a reverse order of lipid oxidation
22 rates between fermented milk products fortified with neat MGK and MPN (as shown in
23 Figure 2b). Fermented milk product fortified with neat MGK had the highest level of 1-
24 penten-3-ol (one of the volatiles derived from n-3 PUFA oxidation) during 28 days storage
25 followed by fermented milk product fortified with neat MPN, whereas fermented milk
26 product fortified with neat fish oil had the lowest level. However, by judging from the initial
27 level of 1-penten-3-ol and the actual increment in its concentration in both marine PL

1 fortified products (80.5 ng/g increment in MPN vs 28 ng/g increment in MGK), a higher
2 degree of lipid oxidation was actually observed in fermented milk product fortified with neat
3 MPN. In addition, the finding that lipid oxidation in fermented milk product fortified with
4 neat MPN was highest was further confirmed by the data for 2-ethylfuran (another n-3
5 derived volatile, data not shown). Thus, the volatiles data were in agreement with PV data
6 and lipid oxidation in fortified products was thus ranked as follows: neat MPN fortified
7 product > neat MGK fortified product > neat fish oil fortified product.

8 This order of lipid oxidation could at least partly be explained by a higher total
9 tocopherol content in product fortified with neat fish oil followed by neat MGK, and MPN
10 had the lowest level of tocopherol (Figure 2c). The total tocopherol content in fortified
11 product correlated with the total tocopherol content in the marine lipids used for fortification.
12 Even though PL was shown to have protective effect against lipid oxidation as reported by
13 several studies [33, 35], this was not the case for two marine PL sources used in the present
14 study. The oxidative stability of marine PL not only was influenced by the level of PL, but
15 also the quality and overall chemical composition of marine PL as previously reported [19].
16 The level of antioxidants, impurities, namely transition metals and initial hydroperoxides in
17 marine PL were found to influence the protective effect of marine PL. Therefore, the lower
18 oxidative stability in marine PL than fish oil could be explained by a higher level of initial
19 hydroperoxides in marine PL. For instance, PV of 1.07meq/Kg and 1.11 meq/Kg were found
20 in MGK and MPN, respectively as compared to PV of 0.16 meq/Kg in fish oil. Furthermore,
21 the better oxidative stability in fermented milk product fortified with MGK than that of MPN
22 was attributed to the better quality of MGK. MGK had a lower level of iron, a higher level of
23 PC, tocopherol and it had additional antioxidant (astaxanthin) as shown in Table 1. The
24 present of 50 ppm esterified astaxanthin in MGK might provide additional protection to
25 fortified products than MPN. In addition, the presence of significant amount of PL and α -

1 tocopherol together might be another reason for better oxidative stability of MGK than MPN
2 [3, 4]. In addition, our previous study also showed that stabilization of PL dispersion was due
3 to the presence of α -tocopherol [36].

4 Fermented milk product fortified with 50% and 10% MPN emulsions showed
5 significantly larger PV and 1-penten-3-ol increments over 28 days of storage as compared to
6 those of the corresponding MGK emulsions (Figure 2a & Figure 2b). Similar to the findings
7 for products fortified with neat MPN or MGK, fermented milk products with MPN emulsions
8 were thus more oxidized than MGK emulsions and this observation was further confirmed by
9 an appreciable decrease in total tocopherol content in both products fortified with MPN
10 emulsion from 14 days storage to 28 days storage (Figure 2c). This difference in oxidative
11 stability was most likely due to the same reasons as those mentioned for the fermented milk
12 products containing neat MPN and MGK (different contents of pro-oxidants and
13 antioxidants).

14 Interestingly, the rank order of lipid oxidation in fermented milk products prepared
15 with pre-emulsified marine PL was reversed as compared to that of the emulsion system it-
16 self (compare Fig. 1a and b vs Fig. 2a and b). There may be different explanations to this
17 phenomenon. The solubility of iron increases with decreasing pH. As previously mentioned,
18 the MPN preparation had the highest content of iron, and its solubility was higher in the
19 fermented milk product system than in the parent emulsion as the fermented milk had an
20 acidic pH compared to the neutral pH of the original marine PL emulsion. The solubilized
21 iron could enhance the decomposition of hydroperoxides to form more free radicals and
22 could also induce lipid oxidation through fast fixation of positively charged iron to negatively
23 charged PL liposomes that were present in the emulsion [37, 38]. The observed difference in
24 oxidative stability of MGK and MPN based systems between the parent PL emulsions and
25 fortified products may therefore be attributed to different behaviour of iron in the two

1 systems. Hence, iron played a greater role in oxidation in the fermented milk product than in
2 the parent emulsion. In emulsion system, other factors such as droplet sizes and structures of
3 particle size most likely had more impact on lipid oxidation than the chemical composition of
4 marine PL.

5 Moreover, it cannot be ruled out that lipid oxidation of marine PL in fermented milk
6 product system might be influenced by other factors such as the presence of antioxidant
7 peptides, free amino acid, low oxygen diffusion in fermented milk product system [39, 40] or
8 synergism between PL and antioxidant peptides. Thus, antioxidants such as α -tocopherol,
9 astaxanthin, ethoxyquin might behave differently due to different pH, concentration and
10 interactions with different compounds in the fermented milk product system. In the present
11 study, particle size distribution (PSD) and viscosity had minimal impact on lipid oxidation in
12 the fermented milk product system. Thus, both plain and fortified products showed a similar
13 monomodal PSD, with a peak at 10 μ M (data not shown). Furthermore, there was no
14 significant difference ($p > 0.05$) in viscosity between fermented milk products fortified with
15 neat marine lipids and pre-emulsified marine PL. In addition, lipid content of pre-emulsified
16 marine PL (10 % or 50 % emulsion) did in general not cause significant differences ($p >$
17 0.05) in lipid oxidation between the two groups of fermented milk products fortified with
18 these emulsions. This showed that the lipid content of pre-emulsified marine PL is not an
19 important factor in lipid oxidation of marine PL fortified products.

20 In addition to the above mentioned n-3 derived volatiles, volatile such as 2-heptanol
21 was also observed in both plain and fortified products. A drastic increase of this volatile at 28
22 days of storage was observed (data not shown). This phenomenon might indicate microbial
23 growth in the fermented milk products as also previously reported by Jacobsen and
24 colleagues [41]. The microbial growth in fermented milk products was confirmed by the

1 detection of pink yeast in fermented milk products with concentrations ranging between 10^5
2 CFU/g and 10^6 CFU/g at 28 days of storage.

3

4 **Sensory evaluation of marine PL fortified fermented milk product**

5 Sensory evaluation was carried out on both plain and fortified products except the fermented
6 milk products fortified with neat marine PL (MPN and MGK). The fishy and other
7 unpleasant flavours were already pronounced in these fermented milk products at the start of
8 the experiment. This is because a higher percentage of marine PL used for neat oil
9 fortification and this led to a higher level of off flavour. In neat oil fortification, 1 g of marine
10 PL was incorporated into 100 g fermented milk products, whereas only 0.5 g of marine PL in
11 the pre-emulsified form (together with 0.95 g of fish oil) was incorporated into 100 g of
12 fermented milk product. In the present study, the pattern of three odour developments,
13 namely sourness, fishiness and rancidness, were almost similar to the corresponding flavours.
14 Thus, only the data for flavour development are shown for discussion (Table 3). Sourness of
15 the fortified products was not affected by the incorporation of marine lipids either in neat or
16 pre-emulsified forms (data not shown). There were no significant differences in sourness
17 between plain and fortified products throughout 21 days of storage.

18 Fortified products seemed to have higher intensity of fishy flavour than plain
19 fermented milk product at all time intervals (Table 3). Fermented milk products fortified with
20 pre-emulsified MPN (10%) showed a higher intensity of fishy flavour than that of pre-
21 emulsified MGK (10% and 50%), especially at day 14 and 21 days of storage. As shown in
22 Table 3, storage increased the intensity of fishy flavour of fermented milk products fortified
23 with pre-emulsified marine PL (10% MPN and 10% MGK). In terms of rancidness (Table 3),
24 significant differences were observed between plain and fortified products (either fortified
25 with neat fish oil or pre-emulsified marine PL). Even though there was no significant

1 increment in rancidness of products fortified with pre-emulsified MPN during storage, the
2 intensity of rancidness already high in these fortified products at 0 days. In summary,
3 incorporation of marine lipids (either fish oil or marine PL) did not affect the sourness of the
4 fortified products, but increased the fishiness and rancidness. The order of intensity for fishy
5 and rancid flavours in fortified products was as follows: product fortified with 10% and 50%
6 MPN emulsion > product fortified with 10% and 50% MGK emulsions > product fortified
7 with neat fish oil > plain fermented milk product. The result obtained from sensory
8 evaluation was thus in agreement with the results from PV and secondary volatile
9 measurements.

10

11 **CONCLUSION**

12 In this study, fortification of fermented milk product with a mixture of fish oil and marine PL
13 did not provide better oxidative stability and sensory quality as compared to fortification with
14 neat fish oil as we had hypothesized. This phenomenon was most likely due to the quality of
15 marine PL used for emulsion preparation and fortification. The order of oxidative stability of
16 marine PL in emulsion systems was different from that in fermented milk products fortified
17 with the same emulsions most likely due to the different behaviour of iron in the different
18 systems. Although the lipid content of the emulsions (10 % vs 50 %) affected the lipid
19 oxidation in parent emulsions, the lipid content did not affect the lipid oxidation in fermented
20 milk products fortified with the same emulsions. The oxidative stability of marine lipids
21 fortified fermented milk products as determined by PV and volatile measurement was ranked
22 as follows: neat or pre-emulsified MPN fortified products < neat/pre-emulsified MGK
23 fortified products < fish oil fortified products. This order of lipid oxidation was in agreement
24 with sensory data, which showed that the intensity of fishy or rancid flavour was highest in
25 products fortified with MPN emulsions followed by products fortified with MGK emulsions

1 and product fortified with neat fish oil was the least oxidized. In conclusion, the oxidative
2 stability and sensory quality of marine PL fermented milk products varied depending on the
3 quality of marine PL used for their fortification. The marine PL used in the present study
4 were commercially available preparations and their quality was comparable to other
5 commercial marine PL preparations applied in our previous studies (Lu et al., 2012a). Taken
6 together the results thus demonstrated that further improvement of the quality of marine PL is
7 necessary before they can be used for food fortification.

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9

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15 **REFERENCES**

- 16 1. Peng JL, Larondelle Y, Pham D, Ackman RG, Rollin X (2003) Polyunsaturated fatty
17 acid profiles of whole body phospholipids and triacylglycerols in anadromous and
18 landlocked Atlantic salmon (*Salmo salar* L.) fry. *Comp Biochem Phys B* 134: 335-
19 348
20
21 2. Wijendran V, Huang MC, Diao GY, Boehm G, Nathanielsz PW, Brenna JT (2002)
22 Efficacy of dietary arachidonic acid provided as triglyceride or phospholipid as
23 substrates for brain arachidonic acid accretion in baboon neonates. *Pediatr Res* 51:
24 265-272
25
26 3. Cho SY, Joo DS, Choi HG, Nara E, Miyashita K (2001) Oxidative stability of lipids
27 from squid tissues. *Fish Sci* 67: 738-743
28
29 4. Moriya H, Kuniminato T, Hosokawa M, Fukunaga K, Nishiyama T, Miyashita K
30 (2007) Oxidative stability of salmon and herring roe lipids and their dietary effect on
31 plasma cholesterol levels of rats. *Fish Sci* 73: 668-674
32
33 5. Ierna M, Kerr A, Scales H, Berge K, Griinari M (2010) Supplementation of diet with
34 krill oil protects against experimental rheumatoid arthritis. *BMC Musculoskelet*
35 *Disorders* 11: 136
36

- 1 6. Narayan B, Miyashita K, Hosakawa M (2006) Physiological effects of
2 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) - A review. *Food Rev*
3 *Int* 22: 291-307
4
- 5 7. Lu FSH, Nielsen NS, Timm-Heinrich M, Jacobsen C (2011) Oxidative stability of
6 marine phospholipids in the liposomal form and their applications. *Lipids* 46: 3-23
7
- 8 8. Trautwein EA (2001) n-3 Fatty acids – physiological and technical aspects for their
9 use in Food. *Eur J Lipid Sci Technol* 103:45-55
- 10 9. FDA (2008a) U. S. Food and Drug Administration, Center for Food Safety and
11 Applied Nutrition, Office of Food Additives Safety: Agency Response Letter: GRAS
12 Notice No. GRN 000226, January 3
13
- 14 10. FDA (2008b) U. S. Food and Drug Administration, Center for Food Safety and
15 Applied Nutrition, Office of Food Additives Safety: Agency Response Letter: GRAS
16 Notice No. GRN 000242, October 14
17
- 18 11. FDA (2011) U. S. Food and Drug Administration, Center for Food Safety and
19 Applied Nutrition, Office of Food Additives Safety: Agency Response Letter: GRAS
20 Notice No. GRN 000371, July 22
21
- 22 12. Pietrowski BN, Tahergorabi R, Matak KE, Tou JC, Jaczynski J (2011) Chemical
23 properties of surimi seafood nutrified with ω -3 rich oils. *Food Chem* 129: 912-919
24
- 25 13. Kassis NM, Gigliotti JC, Beamer SK, Tou JC, Jaczynski J (2011) Characterization of
26 lipids and antioxidant capacity of novel nutraceutical egg products developed with
27 omega-3-rich oils. *J Sci Food Agric* 92:66-73
- 28 14. Sedoski HD, Beamer SK, Jaczynski J, Partington S, Matak KE (2012) Sensory
29 evaluation and quality indicators of nutritionally enhanced egg products with ω -3 rich
30 oils. *LWT-Food Sci Technol* 47: 459-464
31
- 32 15. Nacka F, Cansell M, Gouygou JP, Gerbeaud C, Meleard P, Entressangles B (2001)
33 Physical and chemical stability of marine lipid-based liposomes under acid
34 conditions. *Colloids Surfaces B* 20: 257-266
- 35 16. Nacka F, Cansell M, Entressangles B (2001) In vitro behavior of marine lipid-based
36 liposomes, influence of pH, temperature, bile salts, and phospholipase A(2). *Lipids*
37 36: 35-42
- 38 17. Mozuraityte R, Rustad T, Storro I (2008) The role of iron in peroxidation of
39 polyunsaturated fatty acids in liposomes. *J Agric Food Chem* 56:537-543
- 40 18. Lu FSH, Nielsen NS, Baron CP, Jacobsen C (2012) Physico-chemical properties of
41 marine phospholipid emulsions. *J Am Oil Chem Soc* 89: 2011-2024
42
- 43 19. Lu FSH, Nielsen NS, Baron CP, Jacobsen C (2012) Oxidative degradation and non-
44 enzymatic browning between oxidized lipids and primary amine groups in different
45 marine PL emulsions. *Food Chem* 135: 2887-2896

- 1
- 2 20. Lu FSH, Nielsen NS, Baron CP, Diehl BWK, Jacobsen C (2013). Impact of primary
- 3 amine group from aminophospholipids and amino acids on marine phospholipid
- 4 stability: Non-enzymatic browning and lipid oxidation. Food Chem (In press)
- 5
- 6 21. Let MB, Jacobsen C, Sorensen AD, Meyer AS (2007) Homogenisation condition
- 7 affects the oxidative stability of fish oil enriched milk emulsions. J Agric Food Chem
- 8 51:1773-1780
- 9
- 10 22. Iverson JS, lang LCS, Cooper MH (2001) Comparison of the Bligh and Dyer and
- 11 Folch methods for total lipid determination in a broad range of marine tissue. Lipids
- 12 36:1283-1287
- 13
- 14 23. International IDF Standard 74 A (1991) Milk and milk products: determination of the
- 15 iron content. Brussels: International Dairy Federation.
- 16
- 17 24. Shantha NC, Decker EA (1994) Rapid, sensitive, iron-based spectrophotometric
- 18 methods for determination of peroxide values of food lipids. J AOAC Int 77:421-424
- 19
- 20 25. AOCS (1998) Official method Ce 8-89: Determination of tocopherols and tocotrienols
- 21 in vegetable oils and fats by HPLC. AOCS, Champaign IL, USA
- 22
- 23 26. Timm-Heinrich M, Xuebing X, Nielsen NS, Jacobsen C (2003) Oxidative stability of
- 24 milk drinks containing structured lipids produced from sunflower oil and caprylic
- 25 acid. Eur J Lipid Sci Technol 105: 459-470
- 26
- 27 27. Van Spreekens (1974) The suitability of a modification of Long and Hammer's
- 28 medium for the enumeration of more fastidious bacteria from fresh fishery products.
- 29 Archiv fur Lebensmittelhygiene 25: 213-219
- 30
- 31 28. ISO 8586-1 (1993) Sensory analysis – general guidance for selection, training and
- 32 monitoring of assessors. Part 1: selected assessors. International organization for
- 33 standardization.
- 34
- 35 29. ISO 11035 (1994) Sensory analysis – identification and selection of description for
- 36 establishing a sensory profile by a multidimensional approach. International standard.
- 37
- 38 30. ISO standards 8589 (1988) Sensory analysis – general guidance for design of test
- 39 rooms. International Organization for standardization.
- 40
- 41 31. Saito H, Udagawa M (1992) Application of NMR to evaluate the oxidative
- 42 deterioration of brown fish meal. J Sci Food Agric 58: 135-137
- 43
- 44 32. Mozafari MR, Khosravi-Darani K, Borazan GG, Cui J, Pardakhty A, Yurdugul S
- 45 (2008) Encapsulation of Food Ingredients Using Nanoliposome Technology. Int J
- Food Prop 11: 833-844
- 33 33. Miyashita K, Nara E, Ota T (1994) Comparative-Study on the Oxidative Stability of
- 34 Phosphatidylcholines from Salmon Egg and Soybean in An Aqueous-Solution. Biosci
- 35 Biotechnol and Biochem 58: 1772-1775

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
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19
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29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48

34. McClements DJ, Decker EA (2000) Lipid oxidation in oil in water emulsions: Impact of molecular environment on chemical reactions in heterogeneous food systems. *J Food Sci* 65:1270-1282

35. Boyd LC, Nwosu VC, Young CL, MacMillian L. (1998) Monitoring lipid oxidation and antioxidant effects of phospholipids by headspace gas chromatographic analyses of rancimat trapped volatiles. *J Food Lipids* 5: 269-282

36. Lu FSH, Nielsen NS, Baron CP, Diehl BWK, Jacobsen C (2012) Oxidative stability of emulsions prepared from purified marine phospholipid and the role of α -tocopherol. *J Agric Food Chem* 60: 12388-12396

37. Mei LY, Decker EA, McClements DJ (1998) Evidence of iron association with emulsion droplets and its impact on lipid oxidation. *J Agric Food Chem* 46: 5072-5077

38. Mei LY, McClement DJ, Wu J, Decker EA (1998) Iron-catalyzed lipid oxidation in emulsion as affected by surfactant, pH, NaCl. *Food Chem* 61: 307-312

39. Farvin KHS, Baron CP, Nielsen NS, Jacobsen C (2010) Antioxidant activity of fermented milk product peptides: Part 1-in vitro assays and evaluation in omega-3 enriched milk. *Food Chem* 123: 1081-1089

40. Farvin KHS, Baron CP, Nielsen NS, Otte J, Jacobsen C (2010) Antioxidant activity of fermented milk product peptides: Part 2 - Characterisation of peptide fractions. *Food Chem* 123:1090-1097

41. Jacobsen C, Let MB, Andersen G, Meyer AS (2006) Oxidative stability of fish oil enriched fermented milk products. In: *Seafood research from fish to dish: Quality, safety and processing of wild and farmed fish*. Wageningen academic publishers, Wageningen, Netherlands, pp 71-86

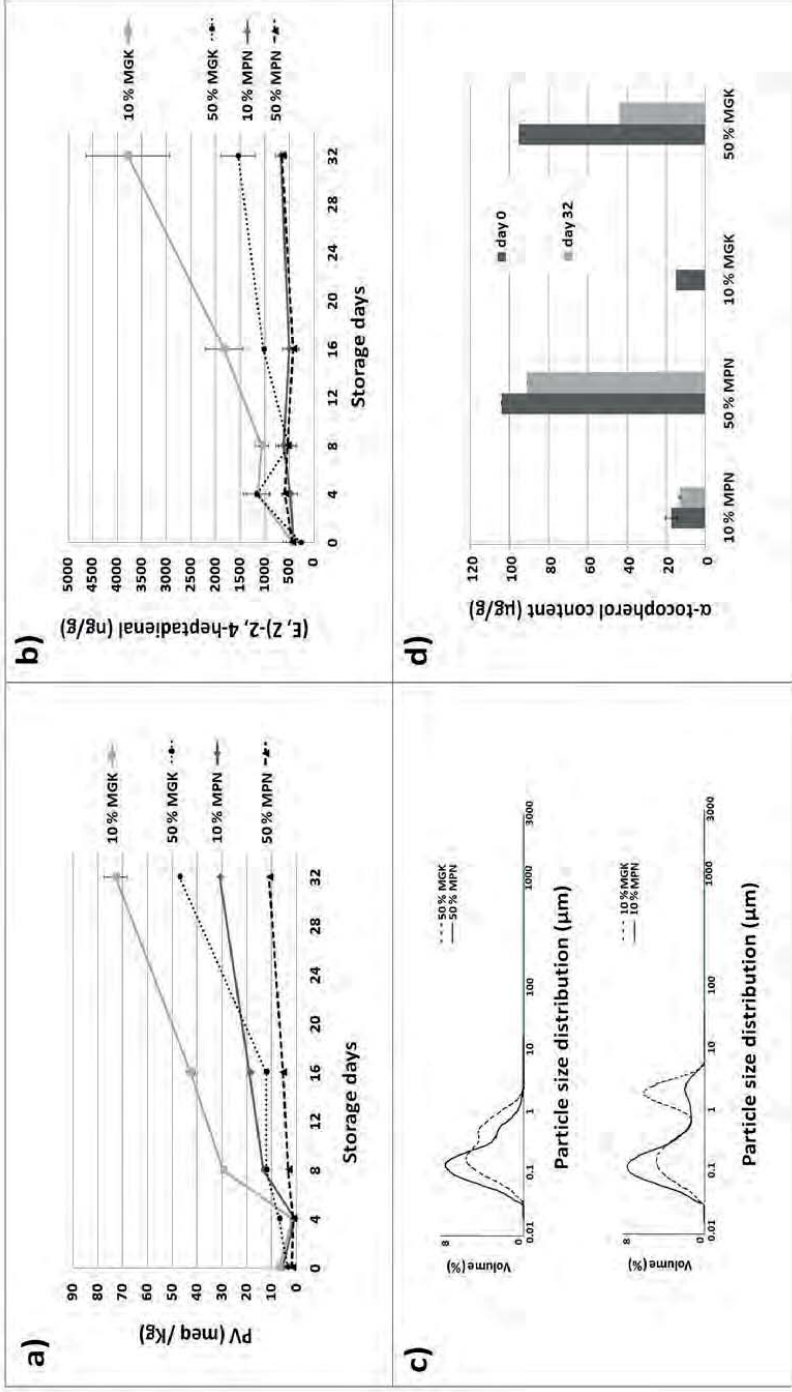


Figure 1 a) The changes of PV in marine PL emulsions during 32 days storage at 5 °C, b) (E, Z) 2, 4-heptadienal increment in marine PL emulsions, c) particle size distribution of marine PL emulsions at day 0 and d) α -tocopherol content in marine PL emulsions before and after 32 days storage. Values are means \pm standard deviation (n = 2) for a) and d), values are means \pm standard deviation (n = 3) for b) and c).

Figure 2 The changes in a) PV, b) 1-penten-3-ol, c) total α -tocopherol content in plain and fortified products during 21 days storage at 5 °C. Values are means \pm standard deviation (n = 2) for a) and c), values are means \pm standard deviation (n = 3) for b).

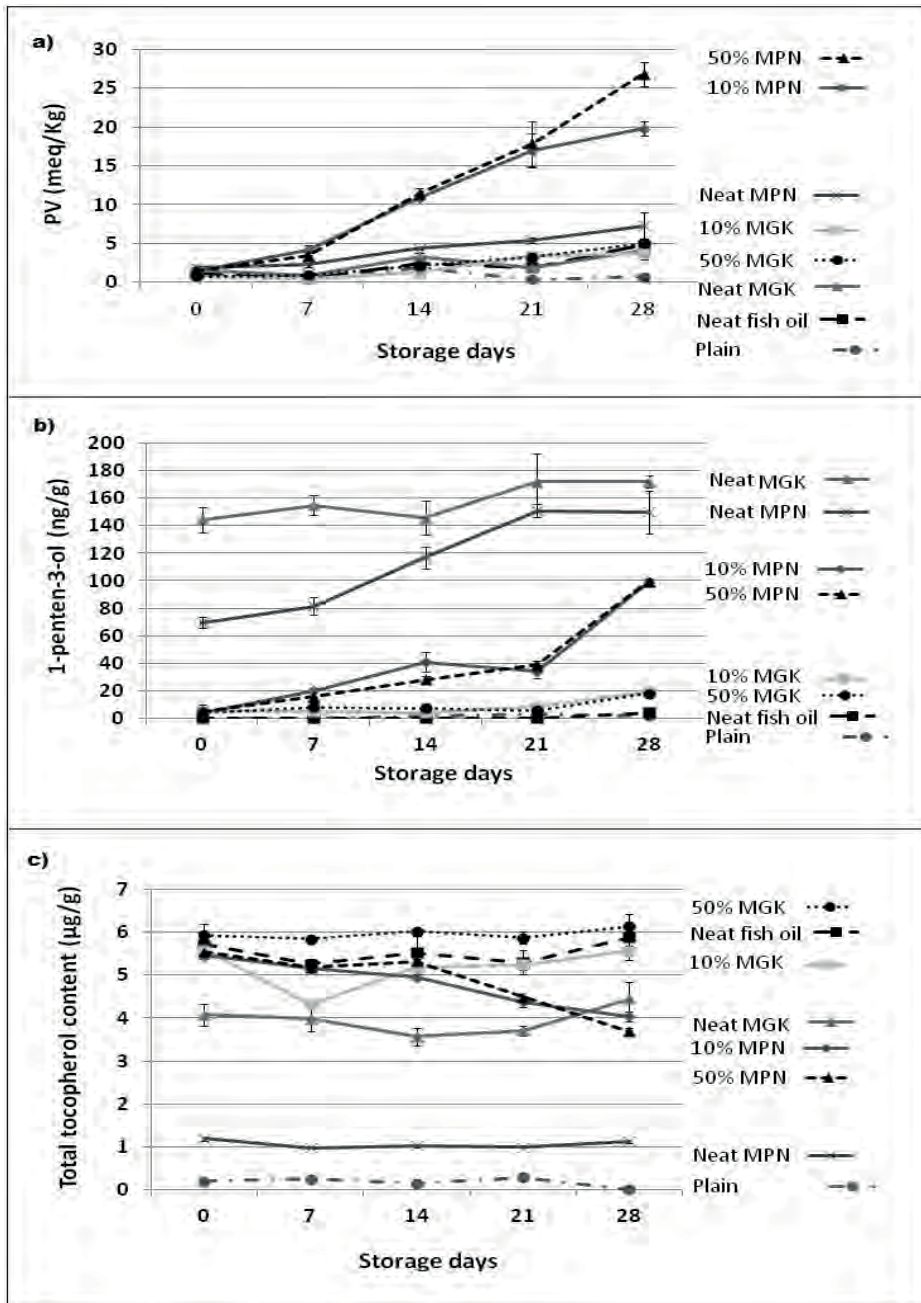


Table 1 : Composition of marine PL used for emulsions preparation

Name	MPN*	MGK*
Sources	sprat fish meal	Antarctic krill <i>Euphausia superba</i>
Total phospholipids (%)	44.34	40.00
<i>Lyso and phosphatidycholine, LPC & PC (%)</i>	21.9	32.0
<i>Phosphatidylethanolamine PE (%)</i>	4.50	4.00
<i>Phosphatidylinositol PI (%)</i>	1.84	2.00
<i>Sphingomyelin SPM (%)</i>	3.50	-
<i>Other phospholipids</i>	12.99	2.00
% Fatty acids composition		
EPA (g /100g)	8.4	11.0
DHA (g /100g)	12.5	7.0
α -Tocopherol (μ g/g)	144 \pm 9.0	466 \pm 11.6
Transition metal, iron (ppm)	6.56	< 1
Peroxide Value (meq/kg)	1.11 \pm 0.01	1.07 \pm 0.01

*MPN also contained < 10 mg/kg ethoxyquin and MGK contained 50 ppm esterified astaxanthin.

Table 2a: Experimental design for marine PL emulsions.

Emulsion formulations	marine phospholipids (%)		fish oil (%)	buffer acetate-imidazole *(%)
	MGK	MPN		
10 % MGK	0.5	-	9.5	90.0
50% MGK	2.5	-	47.5	50.0
10% MPN	-	0.5	9.5	90.0
50% MPN	-	2.5	47.5	50.0

**For fortification, marine PL emulsions were prepared with water instead of buffer.*

Table 2b: Experimental design for products fortification with marine lipids

Formulations	Sources of marine lipids for fortification (g/100g)						
	marine phospholipids (MGK)			marine phospholipids (MPN)			fish oil
	neat	10% emulsion	50% emulsion	Neat	10% emulsion	50% emulsion	
Plain	-	-	-	-	-	-	-
Neat fish oil	-	-	-	-	-	-	1.0
Neat MGK	1.0	-	-	-	-	-	-
Neat MPN	-	-	-	1.0	-	-	-
10% MGK	-	10.0	-	-	-	-	-
50% MGK	-	-	2.0	-	-	-	-
10% MPN	-	-	-	-	10.0	-	-
50% MPN	-	-	-	-	-	2.0	-

Table 3: Sensory attributes of selected flavour of plain and fortified products during 21 days storage at 5 °C using an intensity scale of 1 to 15: a) fishy and b) rancid. Values are means \pm standard deviation (n = 11). Means sharing the same letter in each row (a, b, c, d) and column (x, y) are not significantly different at 5% significant level.

Fishy (flavour)						
Storage days	Plain	Neat fish oil	10%MGK	50%MGK	10%MPN	50%MPN
0	0.56 \pm 1.46 ^{a,x}	1.08 \pm 1.05 ^{ab,x}	2.69 \pm 1.96 ^{abc,x}	2.93 \pm 3.02 ^{bc,x}	4.13 \pm 2.47 ^{c,x}	3.83 \pm 2.35 ^{c,x}
7	0.33 \pm 0.69 ^{a,x}	2.58 \pm 2.18 ^{b,y}	5.14 \pm 2.90 ^{c,y}	3.41 \pm 3.09 ^{bc,x}	4.17 \pm 2.27 ^{bc,x}	3.88 \pm 2.50 ^{bc,x}
14	0.47 \pm 0.89 ^{a,x}	2.43 \pm 1.54 ^{ab,xy}	2.61 \pm 2.48 ^{ab,x}	2.56 \pm 1.54 ^{ab,x}	5.97 \pm 3.27 ^{c,y}	4.38 \pm 2.50 ^{bc,x}
21	0.28 \pm 0.48 ^{a,x}	3.05 \pm 2.09 ^{bc,y}	2.63 \pm 2.10 ^{b,x}	2.54 \pm 2.33 ^{b,x}	5.13 \pm 3.30 ^{c,xy}	4.51 \pm 2.93 ^{bc,x}
Rancid (flavour)						
Storage days	Plain	Neat fish oil	10%MGK	50%MGK	10%MPN	50%MPN
0	0.84 \pm 1.98 ^{a,x}	1.77 \pm 1.84 ^{ab,x}	4.62 \pm 2.73 ^{cd,x}	3.60 \pm 2.68 ^{bc,x}	6.71 \pm 3.06 ^{d,x}	6.87 \pm 2.10 ^{d,x}
7	0.40 \pm 0.97 ^{a,x}	3.46 \pm 2.47 ^{b,y}	7.62 \pm 2.00 ^{c,y}	4.63 \pm 2.46 ^{b,xy}	5.10 \pm 2.35 ^{bc,x}	5.50 \pm 2.70 ^{bc,x}
14	0.68 \pm 1.13 ^{a,x}	3.73 \pm 2.08 ^{b,y}	5.30 \pm 3.04 ^{bc,x}	5.08 \pm 2.85 ^{bc,xy}	6.55 \pm 3.14 ^{c,x}	5.85 \pm 2.49 ^{bc,x}
21	0.50 \pm 0.97 ^{a,x}	4.49 \pm 2.59 ^{b,y}	4.50 \pm 3.18 ^{b,x}	5.30 \pm 2.28 ^{b,y}	5.95 \pm 2.39 ^{b,x}	6.03 \pm 2.62 ^{b,x}

PAPER VII

Lu, F. S. H., Nielsen, N, S., & Jacobsen, C.

Short Communication

Comparison of two methods for extraction of volatiles from marine PL emulsions.

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Short Communication

Comparison of two methods for extraction of volatiles from marine PL emulsions

F. S. Henna Lu, Nina S. Nielsen and Charlotte Jacobsen

Division of Industrial Food Research, National Food Institute, Technical University of Denmark, Kgs. Lyngby, Denmark

The dynamic headspace (DHS) thermal desorption principle using Tenax GR tube, as well as the solid phase micro-extraction (SPME) tool with carboxen/polydimethylsiloxane 50/30 μm CAR/PDMS SPME fiber, both coupled to GC/MS were implemented for the isolation and identification of both lipid and Strecker derived volatiles in marine phospholipids (PL) emulsions. Comparison of volatile extraction efficiency was made between the methods. For marine PL emulsions with a highly complex composition of volatiles headspace, a fiber saturation problem was encountered when using CAR/PDMS-SPME for volatiles analysis. However, the CAR/PDMS-SPME technique was efficient for lipid oxidation analysis in emulsions of less complex headspace. The SPME method extracted volatiles of lower molecular weights more efficient than the DHS method. On the other hand, DHS Tenax GR appeared to be more efficient in extracting volatiles of higher molecular weights and it provided a broader volatile spectrum for marine PL emulsion than the CAR/PDMS-SPME method.

Keywords: Marine phospholipids / Non-enzymatic browning reaction / Oxidative stability / Pyrrolization / Strecker degradation

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1 Introduction

Marine phospholipids (PL) have a high content of n-3 fatty acids such as eicosapentaenoic acids (EPA) and docosahexaenoic acids (DHA). They are highly susceptible to oxidation, which will lead to formation of volatile oxidation products that are responsible for the undesirable flavors formed in oxidized marine PL. However, measurement of lipid oxidation through simple chemical methods such as peroxide value sometimes give misleading results especially for marine PL or fish meal stored for extended periods of time due to the fast decomposition of hydroperoxides. Likewise, spectrophotometric methods for determination of secondary

oxidation products (e.g. the *p*-anisidine method) may be too insensitive to provide accurate information about lipid oxidation in these lipids. In addition, these methods do not provide any information about the identity and concentration of specific volatile oxidation products [1]. Nowadays, the main techniques used to extract volatile compounds in foods are static headspace, dynamic headspace (DHS) analysis (purge and trap) and solid phase micro-extraction (SPME) techniques. SPME involves sampling of volatiles from the headspace above the sample by a fiber mounted in a syringe like device. The fiber contains adsorbing materials on which the volatiles will be adsorbed. Subsequently the fiber is heated and the volatiles transferred to the GC. On the other hand, DHS involves continuously stripping of the sample with an inert gas flow followed by trapping of the volatiles in a tube containing adsorbing materials such as Tenax[®]. Subsequently, the tube is heated and the volatiles transferred to a cold trap before another heating step and transfer to the GC. By combining both sampling and sample preparation into one step, SPME appears to be a fast, sensitive, solventless, and economical technique for analysis of volatile compounds [2]. It has been used for extraction of volatiles from oils [3] and food emulsions [4]. Nevertheless, the conditions

Correspondence: Dr. C. Jacobsen, Division of Industrial Food Research, National Food Institute, Technical University of Denmark, Søtofts Plads, Building 221, 2800 Kgs. Lyngby, Denmark
E-mail: chja@food.dtu.dk
Fax: +454 588 4774

Abbreviations: CAR, carboxen; DHS, dynamic headspace; PDMS, polydimethylsiloxane; PL, phospholipid; SPME, solid phase micro-extraction

of volatiles extraction including the type of extraction technique should be selected according to whether the interest is to isolate low or high molecular volatile compounds or a combination of both [5]. Kanavouras et al. [5] compared the analysis of volatiles from extra virgin olive oil using DHS or SPME. They found that DHS sampling using a Tenax-TA trap extracted more volatiles than SPME using a fiber of polydimethylsiloxane (PDMS) and divinylbenzene. Although, the SPME technique was faster and simpler than DHS sampling, the latter provided a broader spectrum of volatiles. SPME is usually recommended for the extraction of volatiles only when headspace concentrations of volatiles are relatively low. This is due to the higher molecular competition for adsorption to the fiber at relatively high volatile concentrations and this may affect its sensitivity [6]. The objective of this study was therefore to evaluate the extraction efficiency of the DHS and SPME techniques for the subsequent GC–MS analysis of volatile compounds in marine PL emulsion.

2 Materials and methods

Two different marine PL preparations (LC and MPW) were obtained from PhosphoTech Laboratoires (Saint-Herblain Cedex, France) and Triple Nine (Esbjerg, Denmark), respectively. MPW had approximately 40% PL, 40% triglycerides, 2% cholesterol, 73.4 $\mu\text{g/g}$ α -tocopherol, 20 ppm iron, and initial PV of 0.81 meq/kg; whereas LC had approximately 40% PL, 1% triglycerides, 15% cholesterol, 1464.2 $\mu\text{g/g}$ α -tocopherol, 2 ppm iron and initial PV of 1.75 meq/kg. The chemicals, sodium acetate, and imidazole were obtained from Fluka (Sigma–Aldrich, Buchs, Spain) and Merck (Darmstadt, Germany), respectively. Other solvents were of HPLC grade (Lab-Scan, Dublin, Ireland)

2.1 Preparation of marine PL emulsion

Two formulations of marine PL emulsion (300 mL) were prepared with 10% of MPW or LC, respectively. Emulsions were prepared in two steps; pre-emulsification by using Ultra-Turrax (Ystral, Ballrechten-Dottingen, Germany) and homogenization by a Panda high pressure table homogenizer (GEA Niro Soavi SPA, Parma, Italy) using a pressure of 800 and 80 bars for the first and second stages, respectively. After homogenization, 1 mL of sodium azide (10%) was added to each emulsion (220 g) to inhibit microbial growth. Emulsions were stored in 250 mL blue cap bottles at 2°C in darkness and samples were taken on 0, 4, 8, 16, and 32 days for volatiles measurement.

2.2 Measurement of lipid oxidation

Secondary volatiles in emulsions were measured by (a) carboxen (CAR)/PDMS–SPME–GC/MS and (b) DHS–GC/MS. Both techniques have been optimized to analyze lipid

oxidation in fish oil emulsions in our lab. For CAR/PDMS–SPME techniques, approximately 1 g of emulsion, together with 30 mg of internal standard (4-methyl-1-pentanol in rapeseed oil) was mixed on a whirly mixer for 30 s in a 10 mL vial. The sample was equilibrated for 3 min at a temperature of 60°C, followed by extraction for 45 min at the same temperature while agitating the sample at 500 rpm. Extraction of headspace volatiles was done by 50/30 μm CAR/PDMS SPME fiber (Supelco, Bellefonte, PA, USA) installed on a CTC Combi Pal (CTC Analytics, Waldbronn, Germany). A (CAR/PDMS) fiber was chosen as it was reported by Iglesias et al. [7] and verified by us (unpublished results) to be the most effective fiber for extraction of volatiles from fish oil emulsions. Volatiles were desorbed in the injection port of gas chromatograph (HP 6890 Series, Hewlett Packard, Palo Alto, CA, USA; Column: DB-1701, 30 m \times 0.25 mm \times 1.0 μm ; J&W Scientific, CA, USA) for 60 s at 220°C. The oven program had an initial temperature of 35°C for 3 min, with increment of 3.0°C/min to 140°C, then increment of 5.0°C/min to 170°C and increment of 10.0°C/min to 240°C, where the temperature was held for 8 min. The individual compounds were analyzed by MS (HP 5973 inert mass-selective detector, Agilent Technologies, USA; Electron ionization mode, 70 eV, mass to charge ratio scan between 30 and 250). Degree of lipid oxidation in emulsions was quantified by pentanal, hexanal, and 1-pentanol as volatiles derived from the oxidation of n-6 polyunsaturated fatty acids (PUFA); octanal and nonanal as volatiles derived from oxidation of n-9 MUFA; *E*-2-hexenal, 1-penten-3-one, *Z*-4-heptenal, *E*, *E*-2,4-heptadienal, *E*,*Z*-2,6-nonadienal, 2-ethylfuran, and propanal as volatiles derived from oxidation of n-3 polyunsaturated fatty acids. Calibration curves were made by dissolving the related volatile standards in rapeseed oil followed by dilution to obtain different concentrations (0.1–100 $\mu\text{g/g}$).

For DHS thermal desorption technique, secondary volatiles from 4 g of the selected emulsions were collected by purging the emulsion with nitrogen (150 mL/min) for 30 min at 45°C, using 4-methyl-1-pentanol as the internal standard, and trapped on Tenax GR tubes (Perkin–Elmer, CN, USA) packed with 225 mg Tenax GR (60–80 mesh, Varian, Middelburg, Netherlands). The volatiles were desorbed (200°C) from the trap in an automatic thermal desorber (ATD-400, Perkin–Elmer, Norwalk, CT) and cryofocused on a Tenax GR cold trap. Volatiles were separated by GC (HP 5890 IIA, Hewlett-Packard, Palo Alto, CA) and analyzed by MS (HP 5972 mass selective detector). The oven temperature program was: 45°C held for 5 min, 1.5°C/min to 55°C, 2.5°C/min to 90°C, 12°C/min to 220°C and finally held at 220°C for 4 min. The individual compounds were identified by both MS-library searches (Weley 138 K, John Wiley and Sons, Hewlett-Packard) and by authentic external standards. The individual compounds were quantified through calibration curves made by adding 1 μL of standards to Tenax GR tubes directly. The same external standards

as mentioned earlier were used for quantification. Measurements were made in triplicates on each emulsion. Volatile extraction efficiency for both methods was compared through a number of volatiles detected and quantification of peak area in percentage.

3 Result and discussion

3.1 Comparison of lipid oxidation in marine PL emulsions as observed from CAR/PDMS-SPME versus DHS sampling techniques

Among the volatiles associated with eicosapentaenoic acids and docosahexaenoic acid oxidation from marine PL are 1-penten-3-one, 2-hexenal, (*E, E*)-2,4 heptadienal, and (*E, Z*)-2,6-nonadienal. These volatiles have been characterized as very potent odorants that contribute to the off-flavor in bulk fish oil, fish oil emulsion, and fish oil enriched products [8] and thus they were quantified for assessment of lipid oxidation in marine PL emulsion. After 16 days of storage, these volatile oxidation products were found in both emulsions and the MPW emulsion appeared to be more oxidized irrespective of the method used for extraction (Fig. 1). However, when comparing volatiles data obtained with the two different

extraction methods on day 32, a striking difference was observed. Thus, when CAR/PDMS-SPME was used for extraction it was found that the LC emulsion was more oxidized than the MPW emulsion (Fig. 1a), whereas the opposite was found when the DHS technique was used for comparison (Fig. 1b). This difference could mainly be attributed to an unexpected decrease in concentrations of all lipid derived volatiles in the MPW emulsion between day 16 and 32 day when using the CAR/PDMS-SPME method. A similar decrease was only observed for 2,4-heptadienal when using the DHS method. Previous studies have shown a decrease in concentrations of unsaturated aldehydes such as (*E, E*)-2,4-heptadienal and (*E, Z*)-2,6-nonadienal during storage of oxidized PL due to their participation in non-enzymatic browning reactions. These reactions take place between reactive tertiary or secondary lipid oxidation products consisting of six and seven carbon chain length and a free amine group. Hence, the occurrence of such reactions could explain the decrease in the concentration of (*E, E*)-2,4-heptadienal, which was observed for both extraction methods, but it does not explain the decrease in the concentrations of 2-hexenal and 1-penten-3-one, which was only observed for the CAR/PDMS-SPME method. A possible explanation for this decrease is a fiber saturation problem in CAR/PDMS-SPME analysis.

To further clarify these findings, changes in lipid-derived volatiles in the MPW emulsion during storage when extracted by CAR/PDMS-SPME were further scrutinized (Table 1). Concentrations of all secondary volatile oxidation products increased significantly between 0 and 16 days followed by a dramatic decrease after 32 days for all volatiles except 1-pentanol, which continued to increase. Importantly, a dramatic increase in the concentration of 3-methylbutanal (a Strecker degradation product) between 16 and 32 days storage was found. The finding that CAR/PDMS-SPME data showed a large increment in 3-methylbutanal concentration and a concomitant drastic decrease of other lipid derived volatiles in MPW emulsion after 32 days storage suggests that fiber saturation may indeed be a problem in these emulsions (Table 1). Hence, the greater affinity of CAR/PDMS fibers for low molecular weight volatiles caused the volatiles to compete for the same extraction sites of the CAR/PDMS fiber and it seemed that volatiles with low molecular weight, namely 3-methylbutanal, had displaced compounds with molecular weights similar to itself, e.g. pentenal and those with high molecular weights, namely (*E, E*)-2,4-heptadienal, (*E, Z*)-2,6-nonadienal, etc.

Interestingly, the fiber saturation problem for the CAR/PDMS-SPME method was not observed for the LC emulsion. This might be due to the less complex composition of volatiles in the LC emulsion as compared to that of the MPW emulsion. Based on this observation, it seemed that SPME analysis is a fast and suitable method for marine PL emulsion with a less complex composition of volatiles, whereas DHS Tenax GR is a better choice for marine PL emulsion with a more complex composition of volatiles. However, more

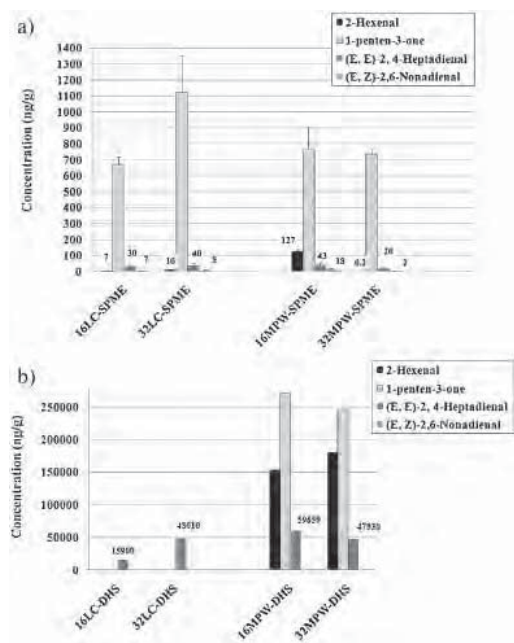


Figure 1. Comparison of n-3 derived volatiles in emulsions (LC & MPW) extracted by two different methods (a) SPME and (b) DHS after 16 days and 32 days storage (SD < 10%).

Table 1. Strecker aldehyde (3-methylbutanal) and lipid derived volatiles in MPW emulsion obtained by SPME extraction

Volatiles (ng/g)	0 days	4 days	16 days	32 days
(<i>E</i>)-2-pentenal (n-3)	427.0	971	2645.0	4.0
1-Penten-3-one (n-3)	295.0	796.6	761.8	489.4
(<i>Z</i>)-4-heptenal (n-3)	4.2	12.3	40.5	0.3
(<i>E, E</i>)-2,4-heptadienal (n-3)	21.5	19.3	42.9	20.4
(<i>E, Z</i>)-2,6-nonadienal (n-3)	10.6	12.4	18.0	2.4
2-Ethylfuran (n-3)	114.2	288.7	449.5	11.2
Propanal (n-3)	263.8	382.6	1533.0	12.0
Hexanal (n-6)	1479.2	1198.0	8215.7	6742.8
Pentanal (n-6)	569.1	501.2	592.4	308.2
1-Pentanol (n-6)	60.7	169.2	1475.7	2226.7
Octanal (n-9)	89.4	117.1	252.4	2.8
Nonanal (n-9)	141.7	131.9	257.5	8.4
3-Methylbutanal (Strecker)	130.0	240.0	851.0	24277.0

studies are required to investigate the possibility of using SPME for volatile analysis in marine PL sample with respect to the suitability of sample matrix. This is because marine PL are more complex than traditional fish oil as they comprise both neutral and polar lipids as well as degradation products from residues amino acids and protein.

3.2 Comparison of volatile extraction efficiency between DHS and CAR/PDMS-SPME techniques

In addition to the lipid derived volatiles, Strecker aldehydes, which are degradation products of amino acids residues [9]

were also found in marine PL emulsions. The following compounds were found: 2-methyl-2-pentenal, dimethyl-disulfide, 3-methylbutanal, dimethyltrisulfide, pyridine, 2-methylbutanal, trimethylpyrazine, and 3-ethyl-2,5-diethylpyrazine as shown in Fig. 2. In total, 22 volatile compounds were extracted in MPW emulsion after 32 days storage by DHS whereas only 14 volatile compounds were extracted by the CAR/PDMS-SPME technique with the experimental conditions used (Fig. 2a). This phenomenon may not only be due to the differences in extraction principles, but also due to the fiber saturation problem in CAR/PDMS-SPME as mentioned earlier. The finding that the DHS method

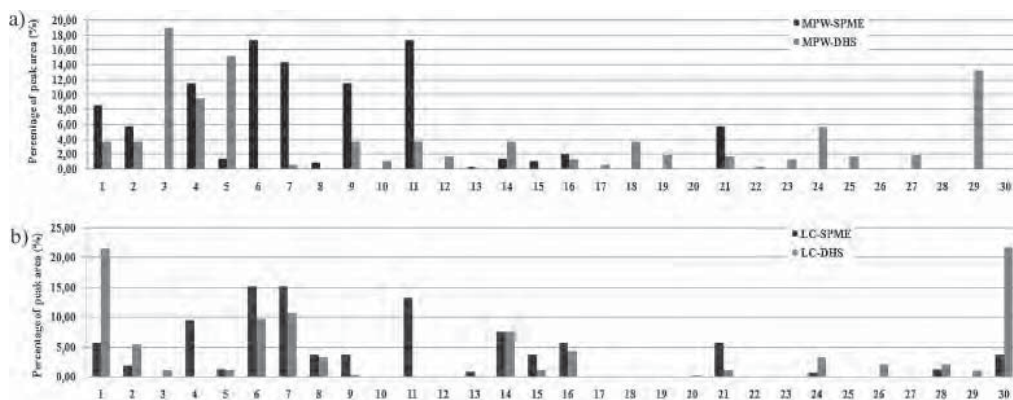


Figure 2. Comparison of main volatiles in marine PL emulsions after 32 days storage (a) MPW and (b) LC by using DHS-GC/MS and SPME-GC/MS methods. (1) 3-Methylbutanal, (2) 2-methylbutanal, (3) 1-penten-3-ol, (4) 1-penten-3-one, (5) pentanal, (6) 2-methyl-2-pentenal, (7) dimethyldisulfide, (8) pyridines, (9) 1-pentanol, (10) (*Z*)-2-penten-1-ol, (11) hexanal, (12) (*E*)-2-hexenal, (13) (*Z*)-4-heptenal, (14) dimethyltrisulfide, (15) trimethylpyrazine, (16) benzaldehyde, (17) (*5Z*)-octa-1,5-dien-3-ol, (18) 7-octen-2-one, (19) octanal, (20) 3-ethyl-2,5-dimethylpyrazine, (21) (*E, E*)-2,4-heptadienal, (22) (*Z*)-octenal, (23) nonanal, (24) 2-nonanone, (25) (*E, Z*)-2,6-nonadienal, (26) 1-methoxy-4-(2-propenyl)-benzene, (27) pentadecane, (28) 2-phenylpropenal, (29) 2,6,10,14-tetramethylpentadecane, (30) 1-methoxy-4-(1-propenyl)-benzene.

extracted more volatiles than CAR/PDMS-SPME in agreement with study of Kanavouras *et al.* [5]. The following volatiles were extracted by DHS but not by SPME: 1-penten-3-ol, (*Z*)-2-penten-1-ol, (*E*)-2-hexenal, (*5Z*)-octa-1,5-dien-3-ol, 7-octen-2-one, (*Z*)-octenal, pentadecane and 2,6,10,14-tetramethylpentadecane. Most of these volatiles are monounsaturated alcohols, aldehydes or ketones with medium polarity or they are hydrocarbons of higher molecular weight. However, the DHS technique provided lower extraction efficiency than CAR/PDMS-SPME towards (*Z*)-4-heptenal and volatiles of lower molecular weight namely pyridines and trimethylpyrazine with the experimental conditions used. This observation seemed to be in agreement with the finding from Rivas Canedo *et al.* [10], who also found that both pyrazine and pyridine from cooked beef meat were extracted by SPME but not by DHS. In general, the DHS technique seemed to extract a larger number of volatiles and thus appeared to be more efficient than CAR/PDMS-SPME when extracting volatiles from marine PL emulsions. However, it is suggested to use both techniques if the objective is to obtain a more complete volatile profile for marine PL emulsions.

For LC emulsion, approximately the same number of volatile compounds (17 volatile compounds) were extracted by both techniques. However, a lower number of volatiles were extracted from the LC emulsion by DHS technique as compared to that of MPW emulsion and this showed that the LC emulsion had a less complex composition of volatiles. Similar to the observations in MPW emulsion, the DHS Tenax GR technique appeared to be more efficient in extracting certain volatiles namely, 1-penten-3-ol, 1-methoxy-4-(2-propenyl)-benzene and 2,6,10,14-tetramethylpentadecane in LC emulsion than the CAR/PDMS-SPME technique. On the other hand, volatiles of lower molecular weight namely 1-penten-3-one, hexanal and (*Z*)-4-heptenal were only found in the LC sample when using the CAR/PDMS-SPME technique. These volatiles have relatively higher air to oil partition coefficient and thus they were easily released from o/w emulsion as compared to volatiles of higher molecular weight namely, nonanal and (*E*, *Z*)-2,6-nonadienal [11]. Taken together, the observations from both samples suggested that the CAR/PDMS-SPME technique seemed to be more efficient in extracting volatiles with lower molecular weights whereas the DHS Tenax GR technique was more efficient in extracting volatiles with higher molecular weights with the experimental conditions used in this study. This phenomenon might be due to the experimental condition used for DHS Tenax GR techniques. This technique has been optimized to collect more high molecular weight volatiles such as 2,4-heptadienal and 2,6-nonadienal and this might have caused the loss of some low molecular weight volatiles as they can “break through” the Tenax material due to the long extraction time. However, the better extraction efficiency of DHS Tenax GR for 1-penten-3-ol of low molecular weight is unexplainable. Thus, future studies are required

to get a better understanding of the relationship between volatile extraction efficiency, the polarity and volatility of the volatile compounds, the partition coefficient between the fiber/Tenax coating materials and volatile compounds as well as air to oil partition coefficient between headspace and samples.

4 Conclusions

In this study, it was found that the DHS Tenax GR and CAR/PDMS-SPME techniques provided different volatile profiles for marine PL emulsions. With the experimental conditions used in the present study, the DHS Tenax GR technique was more sensitive in extracting the volatiles of higher molecular weights and provided a broader spectrum of volatiles. On the hand, the CAR/PDMS-SPME techniques was more sensitive in extracting the volatiles of lower molecular weight. Moreover, even though the CAR/PDMS-SPME technique is a fast method to analyze marine PL emulsions, it should only be used for samples with a less complex matrix as fiber saturation problems might be encountered when analyzing complex food systems. Further studies are needed to confirm whether similar fiber saturation problems will be encountered when using other fiber types than CAR/PDMS.

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References

- [1] Saito, H., Udagawa, M., Application of NMR to evaluate the oxidative deterioration of brown fish meal. *J. Sci. Food Agric.* 1992, 58, 135–137.
- [2] Arthur, C. L., Pawliszyn, J., Solid phase microextraction with thermal desorption using fused silica optical fibres. *Anal. Chem.* 1990, 62, 2145–2148.
- [3] Doleschall, F., Recseg, K., Kemeny, Z., Kovari, K., Comparison of differently coated SPME fibres applied for monitoring volatile substances in vegetable oils. *Eur. J. Lipid Sci Technol.* 2003, 105, 333–338.
- [4] Fabre, M., Aubry, V., Guichard, E., Comparison of different methods: Static and dynamic headspace and solid phase microextraction for the measurement of interactions between milk proteins and flavor compounds with an application to emulsions. *J. Agric. Food Chem.* 2002, 50, 1597–1601.
- [5] Kanavouras, A., Kiritsakis, A., Hernandez, R. J., Comparative study on volatile analysis of extra virgin olive oil by dynamic headspace and solid phase micro-extraction. *Food Chem.* 2005, 90, 69–79.
- [6] Robert, D. D., Pollien, P., Milo, C., Solid-phase microextraction method development for headspace analysis of volatile flavor compounds. *J. Agric. Food Chem.* 2000, 48, 2430–2437.

- [7] Iglesias, J., Lois, S., Medina, I., Development of a solid-phase microextraction method for determination of volatile oxidation compounds in fish oil emulsions. *J. Chromatogr. A* 2007, 1163, 277–287.
- [8] Jonsdottir, R., Bradadottir, M., Arnarson, G. O., Oxidatively derived volatile compounds in microencapsulated fish oil monitored by Solid-phase Microextraction (SPME). *J. Food Sci.* 2005, 70, 433–440.
- [9] Zamora, R., Nogales, F., Hidalgo, F. J., Phospholipid oxidation and nonenzymatic browning development in phosphatidylethanolamine/ribose/lysine model systems. *Eur. Food Res. Technol.* 2005, 220, 459–465.
- [10] Rivas-Canedo, A., Juez-Ojeda, C., Nunez, M., Fernandez-Garcia, E., Volatile compounds in ground beef subjected to high pressure processing: A comparison of dynamic head-space and solid-phase microextraction. *Food Chem.* 2011, 124, 1201–1207.
- [11] Haahr, A. M., Bredie, W. L. P., Stahnke, L. H., Jensen, B., Refsgaard, H. H. F., Flavour release of aldehydes and diacetyl in oil/water systems. *Food Chem.* 2000, 71, 355–362.

National Food Institute
Technical University of Denmark
Mørkhøj Bygade 19
DK - 2860 Søborg

Tel. 35 88 70 00
Fax 35 88 70 01

www.food.dtu.dk

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