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

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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 studies[10] 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-workers[12] 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 phenomenon 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 study[14] 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-workers[5] and Schneider and Løvaas[15] with a few modifications. According to Schneider and Løvaas,[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 Løvaas.[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 Iatroscan 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 dispersions contain mainly liposomes, which have a particle size of 0.1 μm 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-methylbutanal content through SPME-GC-MS. To study the PL pyrrolization in marine PL dispersions, the content of pyrroles and color changes of marine PL dispersions were determined before and after 32 days of storage.

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

Charaterization 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 × 150 mm, i.d. = 3 μm; 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 Iatroscan 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 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. 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 × 2 mL of choloroform and 2-propanol (ratio 2:1) was used to elute the neutral lipid fraction (NL), whereas 3 × 2 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 IDF[20] 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 ^{31}P NMR. PL composition of purified marine PL dispersions was determined through ^{31}P 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)-1*H*-pyrrole (this standard gives absorbance at 570 nm). The pyrrole concentration is thus given as millimoles of 1-(4-methoxyphenyl)-1*H*-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 Clydescale:[24] YI = $142.86b^*/L^*$. 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
sources	sprat fish meal	MPW after acetone precipitation
total phospholipids (%)	**41.50**	**66.23**
phosphatidycholine, PC (%)	18.30	21.34
phosphatidylethanolamine, PE (%)	4.70	9.21
phosphatidylinositol, PI (%)	2.10	2.76
sphingomyelin, SPM (%)	–[a]	–
lysophosphatidycholine, 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. [b]ND, 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]

	MPW (before)		AP (after)
fatty acid	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

[a]Values are means (n = 2, standard deviation < 5%). [b]Unidentified fatty acids.

Table 4. Comparison of Phospholipid Content in AP Dispersions before and after 32 Days of Storage at 2 °C by ^{31}P 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

[a]Only 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

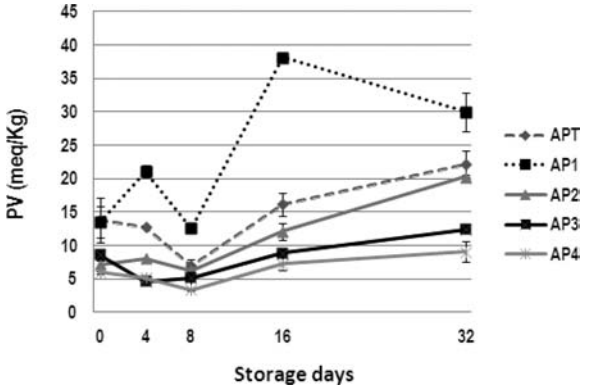

Figure 1. Measurement of PV in AP dispersions during 32 days of storage at 2 °C. Values are the mean ± standard deviation ($n = 2$).

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

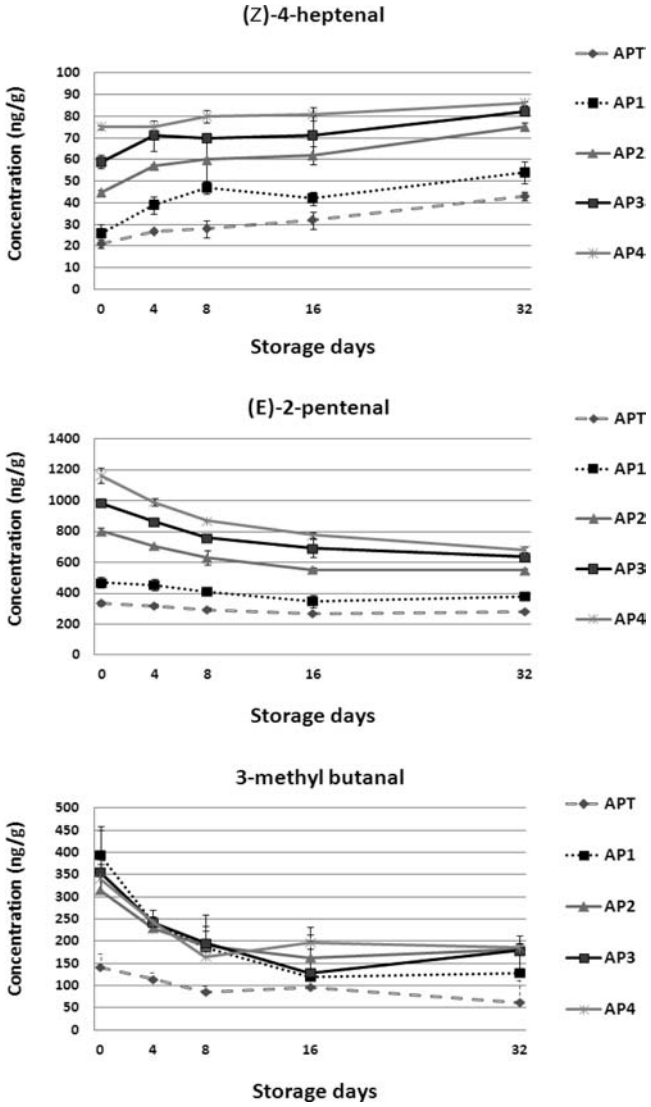

Figure 2. Measurement of (a) (*Z*)-4-heptenal, (b) (*E*)-2-pentenal, and (c) 3-methylbutanal in AP dispersions during 32 days of storage at 2 °C. Values are the mean ± standard deviation ($n = 2$).

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

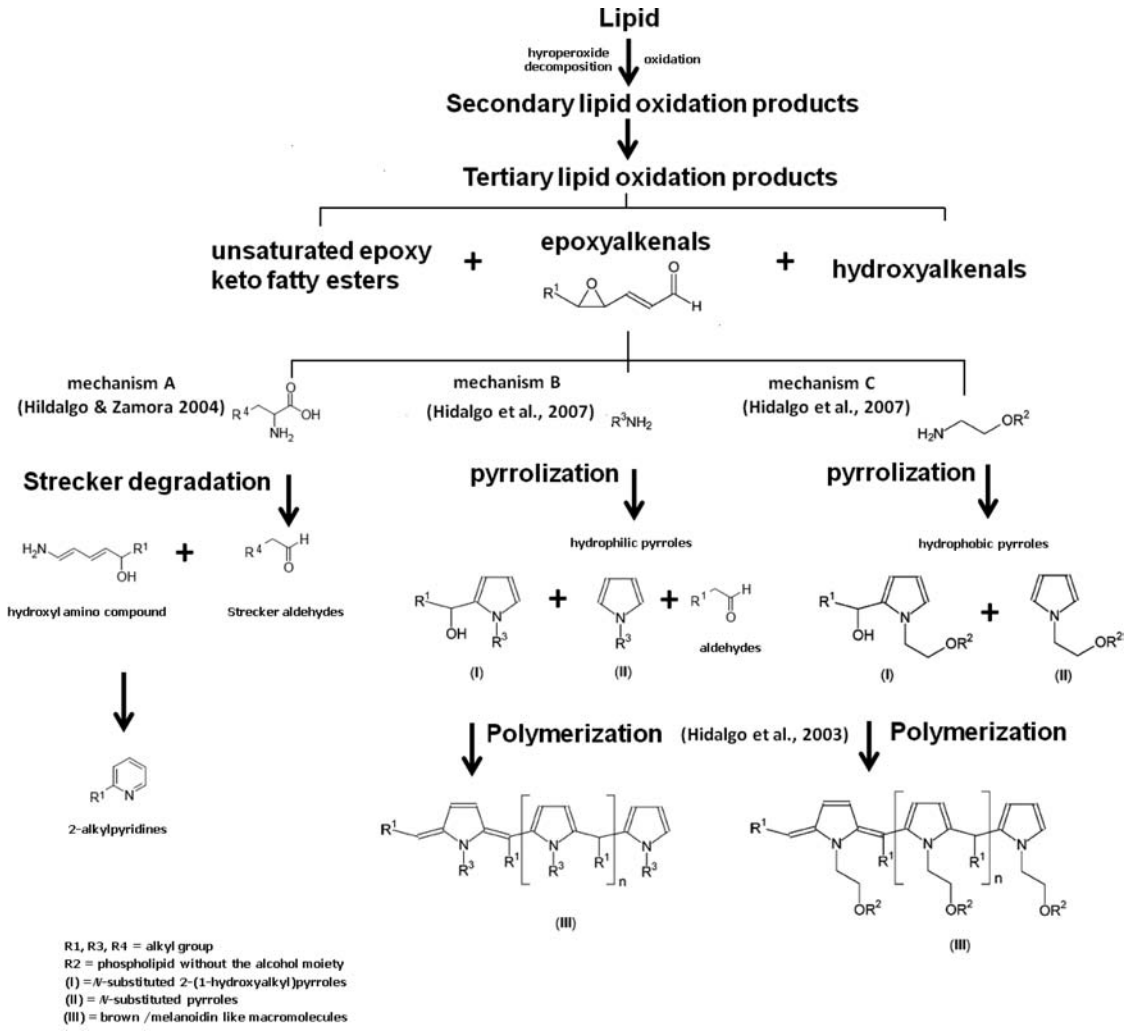

Figure 3. Proposed mechanisms for nonenzymatic browning reactions in marine PL dispersion.

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 study[14] 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-workers[29] 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.

Pyrrolization 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-

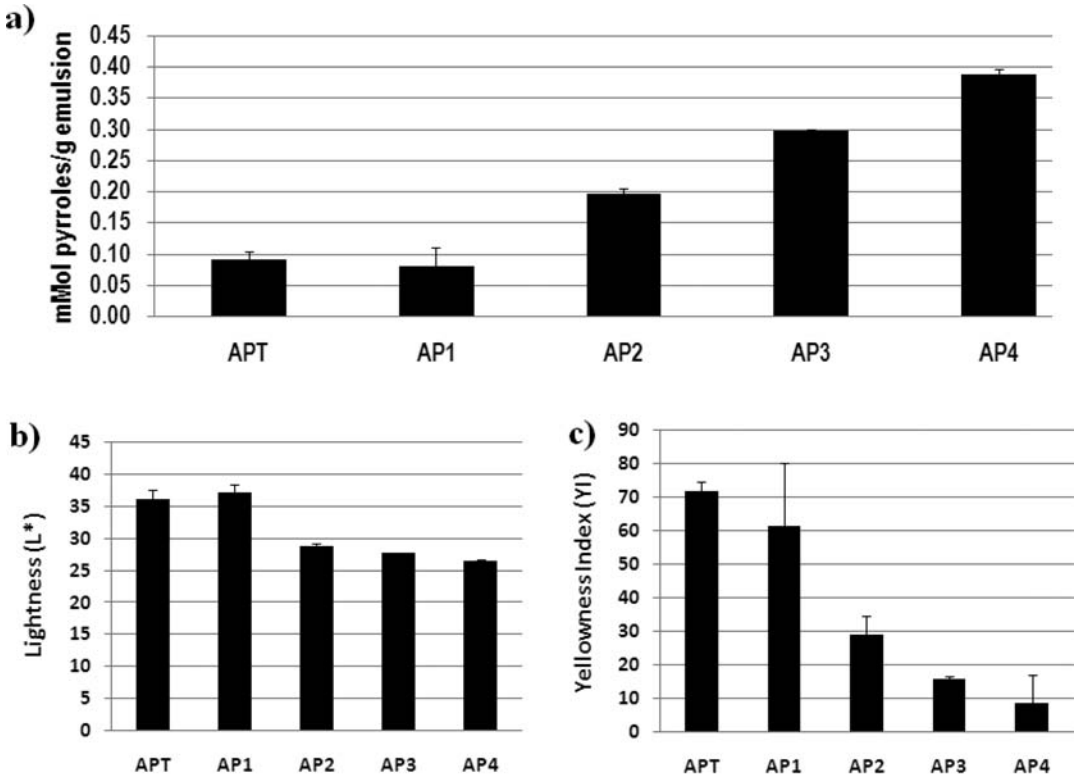

Figure 4. Comparison of (a) pyrrole content (hydrophobic), (b) lightness (L^*), and (c) yellowness index (YI) of marine PL dispersions on day 0. Values are the mean ± standard deviation (n = 2).

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 ^{31}P 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 Nonenzymatic 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 μg/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.

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Notes

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