LC/MS Analysis of Storage-Induced Plasmalogen Loss in Ready-to-Eat Fish

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Abstract

Plasmalogens are functional and oxidation-sensitive phospholipids abundant in fish. Chilling and freezing are common storage methods for maintaining the quality of fish, but their effect on plasmalogen preservation has not been studied. Therefore, plasmalogen loss in ready-to-eat tuna meat during storage under different conditions was investigated. LC/MS was used to analyze the time- and temperature-dependent changes of plasmalogens, which was the most evident for the species with an ethanolamine headgroup and polyunsaturated fatty acyl chains. Moreover, a series of oxidized plasmalogen molecules were identified, and their storage-induced accumulation was observed. Plasmalogen loss was strongly correlated with total lipid oxidation and phospholipid degradation. Repeated freeze-thaw cycles were found to accelerate the loss of plasmalogens, whereas the different thawing methods did not. The present study provides a deeper understanding of changes in lipid nutrients from fish meat during storage and demonstrates the importance of using advanced strategies to maintain food quality.

Keywords

Dietary phospholipids; tuna meat; plasmalogen oxidation; lysophospholipids; freeze-thaw cycles.

1. Introduction

Plasmalogens are a kind of dietary phospholipids with various beneficial effects, such as anti-oxidation (Wu et al., 2019), anti-inflammation (Sejimo et al., 2018), anti-atherosclerosis (Ding et al., 2020), and neuronal protection (Yamashita et al., 2016). Their molecular structures consist of a vinyl-ether-linked chain, an ester-linked fatty acyl chain, and a phosphate headgroup at the *sn*-1, *sn*-2, and *sn*-3 positions of the glycerol backbone, respectively. Depending on the headgroup, plasmalogens are classified into two main types: choline plasmalogen (PlsCho) and ethanolamine plasmalogen (PlsEtn) (Figure 1A).

It is widely believed that intake of bioactive lipids is beneficial for maintaining health, preventing some diseases, and treating certain illnesses. Therefore, the evaluation of quality and quantity of these lipids in foods has gained the attention of scientists. Fish is a well-known source of essential lipids, particularly the major dietary polyunsaturated fatty acyls (PUFA). Plasmalogens are recognized as a "storage pool" of PUFA because their *sn*-2 group is usually a PUFA (J. Wang et al., 2021). PUFA-rich plasmalogens are abundant in aquatic animal food-products, including food-products from fish and shellfish (Wu et al., 2021). Hence, these seafoods have been proposed to help prevent cardiovascular disease and metabolic disorders, e.g., atherosclerosis, nonalcoholic steatohepatitis, and Alzheimer's disease (Ding et al., 2020; Jang et al., 2017; Su et al., 2019).

It is vital to ensure the quality and freshness of fish due to its highly perishable nature. This is particularly the case for ready-to-eat raw fish dishes, such as sushi and sashimi in

Japan and gravlax in Nordic countries. Therefore, after catching, fish are usually kept refrigerated or frozen to extend their shelf-life and increase market accessibility. However, these storage methods induce some changes in the quality of fish, such as color, texture, flavor, and microbial profile (Coombs et al., 2017; Soyer et al., 2010). In terms of chemical components, researchers have demonstrated that the denaturation, rancidity, degradation, and oxidation of proteins and lipids are mainly responsible for the decline in quality and nutritional value of foods (Aguilera Barraza et al., 2015). These changes are exacerbated by inappropriate thawing methods and excessive freeze-thaw cycles (Ali et al., 2015; B. Wang et al., 2020).

Compared with that of conventional evaluation methods, better food quality control can be achieved using the recently proposed “food fingerprinting” strategy that employs an omics approach (Medina et al., 2019). The quality change in fish during storage has been analyzed on the basis of proteins, enzymes, organic acids, biogenic amines, and volatile bases (Prabhakar et al., 2020). In contrast, studies on the lipidome changes remain quite limited. Especially, plasmalogens should be investigated at the molecular level, in order to precisely monitor their easily neglected quality changes and assess the variation in related nutrients. In our previous study, we discovered that plasmalogens were significantly reduced during high-temperature cooking processes (Wu et al., 2020). Although such an issue does not exist for ready-to-eat raw fish, the loss of plasmalogens during preservation remains unknown.

Based on the above considerations, the present study aimed to clarify changes in

plasmalogens in ready-to-eat raw fish meat during storage using liquid chromatography-mass spectrometry (LC/MS). Detailed variations of plasmalogen species with different headgroups (PlsCho and PlsEtn) and various fatty acyls were semi-quantitatively characterized during freezing and chilling under different conditions. The influences of common thawing methods and the number of freeze-thaw cycles were also evaluated and discussed.

2. Materials and methods

2.1. Chemicals

Chloroform, methanol, isopropanol, and water of spectral grade were purchased from Sigma-Aldrich (St. Louis, USA). The plasmalogen standards PlsCho p16:0/17:0 and PlsEtn p16:0/17:0 were chemically synthesized (Wu et al., 2020), while other lipid standards were obtained from Avanti Polar Lipids (Alabaster, USA) and Sigma-Aldrich. A mixed solution containing all internal standards was prepared with methanol and stored at −80 °C until use, and the concentration of each standard in the mixture is listed in Table S1. Unless otherwise specified, other chemical reagents were of analytical grade and acquired from Kanto Chemical Co., Inc. (Tokyo, Japan).

2.2. Sample preparation

Fresh lean tuna meat was purchased from a local provider in Sapporo, Japan, of which the fish were harvested in Hokkaido, Japan. The samples were kept on ice and transported immediately to the laboratory within 20 min. Upon arrival, the samples were cut into uniform

pieces of 4 cm × 4 cm × 2 cm and packaged in polyethylene bags. Unless specified otherwise, fresh tuna meat was designated as the “control sample,” which was used to measure the baseline lipid levels. In all subsequent experiments, the samples were analyzed in quadruplicates (unless specified otherwise).

2.2.1. Freezing and chilling conditions

Frozen tuna samples were stored at −25 °C, −40 °C, or −80 °C for up to 90 days, and lipid extraction was performed at 1, 2, 3, 4, 5, 6, 7, 10, 15, 30, 45, 60, and 90 days. The chilled samples were stored at 4 °C for up to 15 days, and lipid extraction was performed at 1, 2, 3, 4, 5, 6, 7, 10, and 15 days. The frozen samples were subsequently used for lipid extraction.

2.2.2. Thawing methods

Four commonly used thawing methods were compared: refrigerator thawing (RT, 4 °C), air thawing (AT, 20 °C), water immersion thawing (WT, 18 °C), and microwave thawing (MT, 200 W). Fresh samples were frozen at −25 °C for 3 days and then thawed using one of the four methods, till the core temperature reached 4 °C (B. Wang et al., 2020). The required thawing times were: 3 h for RT, 1.5 h for AT, 25 min for WT, and 1 min for MT. The control samples were analyzed directly without thawing.

2.2.3. Freeze-thaw cycles

In each freeze-thaw cycle, the sample was frozen at −25 °C for 21 h and then thawed at 4 °C for 3 h. This treatment was repeated for up to 7 cycles. In these experiments, the control samples were the fresh ones (without freezing or thawing) and those kept frozen for 1 to 7

days (without thawing).

2.3. Lipid extraction

Lipid extraction was carried out according to Folch et al. (Folch et al., 1957) with some modifications (Wu et al., 2020). Briefly, the tuna meat sample was directly homogenized using a kitchen homogenizer (Joyoung Co., Ltd., Hangzhou, China). Then, approximately 50 mg of the homogenized sample was placed in a 1.5 mL Eppendorf® tube and weighed precisely. The homogenate was extracted twice with 900 μL of ice-cold solvent that consisted of chloroform/methanol in 2:1 (v/v) with 0.002% butylated hydroxytoluene and internal standards (for the amount of each internal standard see Table S1). This was followed by vacuum concentration, dissolution of extracts in methanol, and filtration to remove any residue. The whole lipid extraction procedure was completed within 1 h to minimize lipid oxidation or degradation, and the prepared lipid extracts were kept at −80 °C until LC/MS injection.

2.4. LC/MS analysis

The determination of plasmalogens and other lipids was performed using a Shimadzu Prominence HPLC (Shimadzu Corp., Kyoto, Japan) coupled to an LTQ Orbitrap MS (Thermo-Fisher Scientific Inc., San Jose, CA, USA). The chromatographic conditions were as follows: Atlantis T3 C18 column (2.1 mm × 150 mm, 3 μm, Waters, Milford, MA, USA), oven temperature of 40 °C, and flow rate of 200 μL/min. The mobile phases were as follows: 10 mM aqueous ammonium acetate (A), isopropanol (B), and methanol (C). The elution

gradient was as follows: 0–1 min, 40%A+20%B+40%C; 1–5 min, 20%A+50%B+30%C; 5–12 min, 5%A+70%B+25%C; 12–28 min, 3%A+82%B+15%C; 28–36.5 min, 3%A+85%B+12%C; 36.5–37.5 min, returned to the initial condition, followed by 40 min for re-equilibration. For MS detection, the following parameters were applied for both electrospray ionization (ESI)-positive and ESI-negative modes: spray voltage of 3 kV, capillary temperature of 330 °C, and Fourier transform mode resolution power of 30 000. The scanning parameters were based on our previous study (Wu et al., 2020): the high-resolution (HR) MS^1 data were obtained in the range of *m/z* 250–1100 for ESI-positive mode and in *m/z* 220–1650 for ESI-negative mode, while the tandem MS data were acquired using collision-induced dissociation (CID). The collision energy values for MS^2, MS^3, and MS^4 were set at 35.0, 40.0, and 40.0, respectively.

The raw data were processed using the workstation Xcalibur 2.3 (Thermo-Fisher Scientific Inc.). The annotation of plasmalogens and other lipids was based on the retention behavior and HR-MS signals with a tolerance of 5.0 ppm. The annotated lipids were labeled as “lipid class + the number of acyl carbon atoms + the number of acyl double bonds.” The fatty acyl composition of the intact lipid molecular species was determined with the help of MS/MS fragmentation, which was based on comparison with LIPIDMAPS (www.lipidmaps.org) and our in-house library (Z. Chen et al., 2020; Wu et al., 2019, 2020). The semi-quantitation of plasmalogens and other unoxidized lipids was based on the extracted ion chromatogram (EIC) peak areas of the analyte and the IS, according to the following equation:

154 $$Amount_{analyte} = \frac{Peak\ area_{\ analyte}}{Peak\ area_{\ internal\ standard}} \times Amount_{\ internal\ standard}$$

155 Because we lacked internal standards for oxidized lipids, their semi-quantitative
156 measurement was based on calculating the peak area as the analyte intensity.

157

158 **2.5. Measurement of thiobarbituric acid-reactive substances (TBARS) content**

159 TBARS level was measured according to previous studies (Jiang et al., 2019; Li et al., 2019)
160 with some modifications. In brief, 100 mg homogenized sample was mixed with 1 mL acetic
161 acid solution (pH = 4.0, adjusted by NaOH) containing 0.1% EDTA, followed by
162 centrifugation (5000 rpm, 20 min, 4 °C) to obtain the supernatant. Next, 200 μL of the
163 supernatant was reacted with 1 mL of 20 mM thiobarbituric acid (TBA) at 100 °C for 1 h.
164 The reaction was terminated by cooling in an ice bath for 10 min, and then the mixture was
165 separated by centrifugation at 12 000 rpm for 10 min at 4 °C. Fluorescence of the final
166 supernatant was measured at 532 nm using a spectrofluorometer (FP-6500, JASCO, Tokyo,
167 Japan). Malonaldehyde (MDA) prepared from 1,1,3,3-tetraethoxypropane (TEP) was used to
168 obtain the TBARS calibration curve, and the TBARS values were expressed in units of
169 mg MDA/kg sample.

170 **2.6. Statistical analysis**

171 All results were expressed as mean ± standard deviation (SD). Two-tailed Student's t-test and
172 one-way ANOVA (using Tukey's post hoc test) were carried out using GraphPad Prism 8 (La
173 Jolla, CA, USA). $P < 0.05$ was considered significantly different. Correlation analysis and the

following heatmap illustration were performed using *R* 4.1 (R Core Team, 2021). Principal component analysis (PCA) and artificial neural network (ANN) modeling were conducted using JMP 16 pro (SAS Institute Inc., Cary, NC, USA).

3. Results and discussion

3.1. Time- and temperature-dependent loss of plasmalogens during storage

3.1.1. Decrease in the content of total plasmalogens

Representative photographs of tuna meat samples during storage by freezing/chilling are shown in Figure 1B. The content of total plasmalogens, defined as the sum of all the detected intact plasmalogen species (for the identification details see Table S2), was compared at different storage times and temperatures. As shown in Figure 1C, approximately 90% of the total plasmalogens was retained after 90 days of frozen storage under −40 °C and −80 °C. Samples frozen under −25 °C showed a slight decrease (94.5% ± 2.0% of control) in plasmalogen content within the first 5 days, followed by an obvious decline toward the end to reach 68.7% ± 1.9% of the control (i.e., fresh samples without freezing or chilling). Chilling at 4 °C did not preserve the plasmalogens well, since their total amount was only 53.8% ± 2.2% after 15 days. Similar results have been reported for the loss of other fat-soluble nutrients (e.g., fatty acids and certain vitamins) during long-term storage (Karlsdottir et al., 2014; Santos et al., 2012; Zhou et al., 2019). A major issue is temperature variation, which is associated with physiological and biochemical changes in lean meat as well as

194 microbial activity (Ali et al., 2015).

195 **3.1.2. Variation in characteristics of plasmalogen fingerprints**

196 Because plasmalogens include many molecular species, storage-induced variations in their
197 profile need comprehensive investigation. To obtain a general overview, we conducted PCA
198 using the plasmalogen molecular composition as variables for all the samples. There were a
199 total of 198 samples: 52 for each group frozen under −25 °C, −40 °C, or −80 °C; 36 for the
200 group chilled at 4 °C; and 6 for the control group (not frozen or chilled). Most of the variance
201 was adequately explained by the first two principal components (50.6% and 12.3% of the
202 total explained variance). Separate score plots for each storage temperature (Figure 2A) show
203 time- and temperature-dependent clustering. All samples frozen under −40 °C and −80 °C
204 were grouped tightly; those frozen under −25 °C were loosely grouped and showed
205 noticeable divergence after 10 days. Samples chilled under 4 °C displayed migration toward
206 the positive x-axis, which corresponded with storage time from day 4 onward, moving
207 gradually away from the control. These data suggest that a higher storage temperature
208 (particularly chilling at 4 °C) causes more changes in the plasmalogen fingerprint.

209 Figure 2B compares the two plasmalogen types, namely PlsCho and PlsEtn with different
210 headgroups. Both exhibited a decreasing trend during storage. PlsEtn was more unstable than
211 PlsCho under all investigated temperatures, and their difference was especially obvious at
212 higher temperatures. Consistently, in case of the corresponding diacyl glycerophospholipids,
213 the amount of phosphatidylethanolamine (PE) remained significantly lower than that of

214 phosphatidylcholine (PC). Similar studies claimed that PE was more susceptible to hydrolysis
215 or oxidation than PC during the dry curing of meat (Pérez-Palacios et al., 2010; Xu et al.,
216 2008). The present results also suggest the parallel explanation that plasmalogens containing
217 ethanolamine (PlsEtn) suffer more loss during storage than those containing choline
218 (PlsCho).

219 Interestingly, PlsEtn (with a vinyl-ether linkage at *sn*-1) experienced less loss than PE
220 (with an ester linkage at *sn*-1) when stored under −25 °C and 4 °C, even though they share
221 the same headgroup. In contrast, such phenomenon was not found under −40 °C or −80 °C
222 (Figure 2B). One possible explanation is that at higher storage temperatures, phospholipids
223 were partially hydrolyzed by active enzymes, such as lipases and phospholipases (Qinsheng
224 Chen et al., 2017), and more ester linkages mean easier enzymatic reactions. However, such
225 difference according to the linkage was not observed between PlsCho and PC. These results
226 seem to contradict the conventional view that the vinyl-ether linkage serves as a sacrificial
227 antioxidant functional group (Broniec et al., 2011; Morel et al., 2021). Whether there are
228 specific mechanisms that stabilize plasmalogens and how they work are worthy questions for
229 future investigations.

230 Figure 2C compares the fatty acyl compositions across the samples. The levels of PUFAs
231 such as C22:6 and C20:5 were sharply depleted when stored for longer times and under
232 higher temperatures. It is noted that there were higher PUFA levels in PlsEtn than in PlsCho
233 (e.g., 68.5% ± 0.8% vs. 23.6% ± 1.0% for C22:6, $P < 0.001$), which might explain the

different stabilities of PlsEtn and PlsCho (Pérez-Palacios et al., 2010; Xu et al., 2008). According to these results, the stability of plasmalogen species depends on both the headgroup and the fatty acyl at the *sn*-2 position.

3.2. Potential mechanisms of plasmalogen loss during storage

3.2.1. Identification of oxidized plasmalogens

Since plasmalogens are easily oxidizable food nutrients, we were interested to know whether and how they become oxidized during long-term storage, as well as the possible connection between oxidation and plasmalogen loss. To the best of our knowledge, there have been no available libraries for oxidized plasmalogens so far. Therefore, first, we identified a series of oxidized plasmalogen molecules as the oxidation products based on HR-MS signals and tandem MS fragmentation characteristics (Table S3). For instance, the HR-MS^1 data in Figure 3A indicate a signal at *m/z* 810.4937 that corresponds to $C_{43}H_{74}O_{11}NP^-$ (calculated *m/z*: 810.4927, Δppm = 1.23). Considering both the formula (63.9797 Da greater than PlsEtn 38:6) and the retention time (approximately 4 minutes shorter than PlsEtn 38:6) in the corresponding EIC, we assumed the existence of oxidation product PlsEtn 38:6+4[O]. In the following MS^2, compared with that in the intact PlsEtn 38:6 (major fragments: *m/z* 436 as loss of *sn*-2 acyl chain, *m/z* 327 as *sn*-2 $RCOO^-$ ion), there were three major extra ions, namely *m/z* 452, *m/z* 391, and *m/z* 375, suggesting the addition of 1[O] (*m/z* 436 + 16), 4[O] (*m/z* 327 + 64), and 3[O] (*m/z* 327 + 48), respectively. The following MS^3 of *m/z* 452 gave subsequent fragments of *m/z* 391 and *m/z* 255, which were respectively assigned as

[M – H – *sn*-2 acyl chain − ethanolamine]$^-$ and [*sn*-1 epoxy alcohol]$^-$ (or detached as α-hydroxyaldehyde) based on the literature (Stadelmann-Ingrand et al., 2001; Weisser et al., 1997). The product ions of the *m/z* 375 precursor ion exhibited continuous loss of H_2O, suggesting one hydroxyl and one hydroperoxyl group. Therefore, one of the products in the mixture was speculated as PlsEtn p16:0(epo)/22:6(OH)(OOH). Similarly, the MS^3 of *m/z* 391 and the MS^4 further prompted the combination of two hydroxyl and one hydroperoxyl group. Thus, the other product of the isomer was disclosed as PlsEtn p16:0/22:6$(OH)_2$(OOH).

Now, we further discuss the complexity of these oxidized plasmalogens. Taking the molecular species with fatty chain combination 38:6 (identified as p16:0/22:6) as an example, both PlsEtn and PlsCho were found with various oxidation degrees from +1[O] to +4[O], which were respectively classified as epoxides, hydroxides, and hydroperoxides according to the characterized MS fragmentation (Figure S1 – S8). Moreover, the fragment intensity differences indicated that the primary oxidation position for PlsEtn was the double bonds in the *sn*-2 fatty acyls (MS^2 ion intensity: *m/z* 436 > *m/z* 452, *m/z* 343 > *m/z* 327; Figure S1), whereas for PlsCho it was easier to generate epoxy group on the vinyl-ether bond (MS^2 ion intensity: *m/z* 480 > *m/z* 464, *m/z* 327 > *m/z* 343; Figure S5). When two or more oxygen atoms were added into the molecule, the *sn*-1/*sn*-2 + n[O] and the *sn*-1 + [O]/*sn*-2 + (n-1)[O] species were produced without selectivity. Possible reasons might include the proportion of PUFA in the *sn*-2 fatty acyls and the electrical charge on the headgroups.

3.2.2. Oxidation and degradation as the potential mechanisms of plasmalogen loss

Changes in oxidized plasmalogens were assessed for all samples (Figure 3B). The concentrations of both oxidized PlsEtn and oxidized PlsCho increased during storage. Among the four temperatures, −25 °C produced the most drastic accumulation of all plasmalogen oxides, while −40 °C resulted in much lower levels (only 0.3%–27.4% of that under −25 °C). Moreover, there was an even stronger relative reduction in the highly oxidized species at –40 °C (approximately 27% for both PlsEtn+1[O] and PlsCho+1[O], but less than 2% for PlsEtn+4[O] and PlsCho+4[O]). In addition, the plasmalogen oxides emerged at similar times under −25 °C and −40 °C: after 15 days for +1 [O] and after 30 days for +2 to +4 [O]. Importantly, no oxidized plasmalogens were detected at any time in the samples kept under −80 °C. On the 15th day, the samples chilled under 4 °C showed higher levels of oxides than those kept under other temperatures. Also, the peak of oxidized plasmalogens first appeared in the chilled samples on the 7th day, suggesting that these unstable oxides produced under a relatively high temperature would undergo further reactions. The chain reactions of oxidation/peroxidation might affect other lipid components as well as the overall quality of the fish.

We also measured the levels of the oxidation products of other major lipid classes in the tuna meat samples, including triglycerides (TG), diglycerides (DG), free fatty acids (FFA), PC, and PE, according to our previously established LC/MS method (Z. Chen et al., 2020; Wu et al., 2019). The oxidation trends of these lipids were very similar to those of the

293 plasmalogen oxides (shown in Figure 4A). TG, DG, and FFA are known as the main lipid
294 components of lean fish meat, while PC and PE are the major phospholipids. Therefore,
295 oxidation of these lipids induces denaturation and rancidity, critically impacting the quality
296 and nutritional value of the meat (Zhou et al., 2019). Meanwhile, we measured the TBARS
297 levels in these samples (Figure 4A). As secondary products of lipid oxidation, TBARS were
298 found to increase continuously during long-term storage, growing to 3.7-, 7.6-, 2.8-, and 1.2-
299 fold of that of control under 4 °C, −25 °C, −40 °C, and −80 °C, respectively. These findings
300 agree with the measured data for oxidized lipids.

301 Besides oxidation, degradation is another major pathway of plasmalogen loss in biological
302 samples (Engelmann, 2004; Guang et al., 2010). Phospholipids are known to be hydrolyzed
303 into lysophospholipids. For plasmalogens, the degradation products of PlsCho and PlsEtn are
304 lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE), respectively,
305 together with FFA (Qinsheng Chen et al., 2017; Wu et al., 2019). Consequently, their levels
306 were compared to the total amount of every molecular species within the same class
307 (Figure 4B). Similar to the trend observed for oxidation products, the plasmalogen
308 degradation products also accumulated in a temperature- and time-dependent manner.
309 Notably, chilling (4 °C) for 15 days resulted in a larger amount of degradation products than
310 that in all samples stored by freezing (up to 90 days). Lipid degeneration, which consists of
311 oxidation and degradation, is considered the main limiting factor for the shelf-life of cold-
312 stored fish (Tappi et al., 2020). According to our results, extended storage under 4 °C and

313 −25 °C caused significant degeneration of plasmalogens and other related lipids and storage
314 under −40 °C could partially attenuate the degeneration, while storage under −80 °C could
315 effectively inhibit such changes.

316 **3.2.3. Plasmalogen loss as an indicator of lipid degeneration and meat quality**
317 **deterioration**

318 To examine the possible use of plasmalogens as an indicator of fish quality, we analyzed the
319 association between plasmalogen loss and other lipid degeneration indexes assessed in the
320 current study. The Spearman's correlation coefficients were calculated for the samples stored
321 under 4 °C and −25 °C, as summarized in the heatmap of Figure 5A. Significant positive
322 correlations were observed for both temperatures (4 °C, $r > 0.70$; −25 °C, $r > 0.56$),
323 suggesting an agreement between the reduction of intact plasmalogens and the accumulation
324 of oxidized/degraded lipids. It was found that the total plasmalogens and PlsEtn levels
325 correlated better to the lipid degeneration indexes than to PlsCho levels. Considering that
326 TBARS are among the most common indicators of meat oxidation and quality deterioration,
327 we made scatter plots of the TBARS levels and the loss rate of total plasmalogens for
328 samples stored under all four temperatures (Figure 5B). The correlation coefficients were in
329 the following order: −80 °C < −40 °C < −25 °C ≈ 4 °C, indicating that the plasmalogen loss
330 was closely related to meat quality deterioration under commercial freezing and conventional
331 chilling conditions. In addition, a higher storage temperature resulted in a steeper slope of the
332 regression curve, suggesting that plasmalogens may be a more sensitive indicator of meat

quality than TBARS. Thus, the present work indicates the feasibility of a plasmalogen-based quality control strategy.

To provide a global view of plasmalogen loss during long-term storage, we established a non-linear prediction model based on ANN according to our previous method (Wu et al., 2020). ANN is a sophisticated simulation tool that can solve poorly understood problems in food science and technology (Ameer et al., 2017; Aung et al., 2022; Wu et al., 2020). The model was validated by comparing the fitted and measured values in both the training and the validation datasets (Figure S9). Figure 5C shows the predicted amounts of plasmalogens versus the storage temperature and storage time. A temperature between −40 °C and −80 °C is sufficient for preserving the total content of plasmalogens and PlsEtn (with a retention of approximately 90% even after 90 days), while temperatures higher than −40 °C are increasingly risky. In comparison, PlsCho was better preserved during storage: even after 30 days under 0 °C, nearly 75% of PlsCho was present. Overall, −40 °C appears to be a watershed temperature, which is in agreement with the results from a previous study that the state of water fraction and the properties of ice crystals under this optimum temperature were ideal for meat quality attributes (Leygonie et al., 2012). However, for conventional daily storage (chilling or freezing at approximately −20 °C), the content of intact plasmalogens was quite sensitive to the storage time and temperature. For instance, to preserve at least 60% of the total plasmalogens, the fish could be stored at above 0 °C for 11 days or frozen under −10 °C for up to 27 days. To keep more than 80% of the total plasmalogens, the storage times

353 are sharply shortened to 3 days and less than 6 days for 0 °C and −10 °C, respectively.
354 However, if the freezing temperature is reduced to −20 °C, the storage limit could be
355 extended to 14 days. The established model predicts a generally expected pattern, that a
356 shorter storage time and a lower freezing temperature help preserve plasmalogens in stored
357 tuna meat. Furthermore, the model could be a potential strategy for seafood suppliers and
358 consumers to better predict and even control the food quality in terms of functional
359 phospholipid nutrients.

360 **3.3. Effects of thawing on plasmalogen loss**

361 **3.3.1. Different thawing methods had no effect on plasmalogens**

362 After freezing, the subsequent thawing process plays a critical role in the shelf-life of fish
363 products (Ali et al., 2015). To explore the effects of thawing on plasmalogens, we measured
364 the contents of total plasmalogens, PlsEtn, and PlsCho in samples thawed using the four
365 common methods (Figure 6A). Compared to the sample without thawing, none of the thawed
366 samples showed changes in the plasmalogen contents. It should be noted that the current
367 results appear inconsistent with those of some previous studies, which demonstrated that
368 various thawing methods affect the nutritional components (e.g., vitamins and polyphenols)
369 and the degeneration indexes (e.g., TBARS and carbonyl content) (Holzwarth et al., 2012;
370 Xia et al., 2012). This might be explained by the differences in the food samples, their
371 chemical constituents, and the thawing conditions. Nevertheless, our results here suggest that
372 none of the thawing methods caused noticeable plasmalogen loss during single thawing

373 process.

374 **3.3.2. Freeze-thaw cycles accelerate plasmalogen loss**

375 Repeated freeze-thaw cycles are known to accelerate the deterioration of food quality
376 (Qingmin Chen et al., 2018). However, such results have not been reported for plasmalogens.
377 The data in our present study indicated that plasmalogen loss became worse after multiple
378 freeze-thaw cycles (Figure 6B). After two cycles, the total plasmalogen content was
379 significantly reduced compared to that of the corresponding control ($P < 0.01$), and the
380 decline continued until the 7th cycle. At the end of 7 cycles, the freezing-only samples lost
381 10.8% of the total plasmalogens, while those subjected to freeze-thaw cycles lost an extra
382 12.4% ($P < 0.001$ vs. freezing-only samples). PlsEtn exhibited a similar behavior with a
383 higher loss than the freezing-only sample starting at the 2nd cycle, and the final extra loss was
384 12.8%. For PlsCho, the additional loss compared to the control appeared slowly at the 7th
385 cycle (11.9%, $P < 0.05$ vs. freezing-only samples).

386 These findings are partially consistent with those of previous reports that lipid oxidation in
387 meat products became worse after three or more cycles (Ali et al., 2015; Qingmin Chen et al.,
388 2018). However, our results also indicated that for plasmalogens in fish meat, especially
389 PlsEtn, this restriction should be reduced to only one cycle. Besides, because plasmalogens
390 are fairly susceptible to quality deterioration, they might serve as a more sensitive tool for
391 monitoring changes in the freshness of meat due to thawing.

392 Overall, our study revealed important behaviors in the loss of plasmalogens in ready-to-eat

raw fish during storage (including chilling, freezing, and thawing). One study limitation is that the determination of plasmalogen levels was based on a semi-quantitative profiling method by LC/MS, whereas it is desirable to perform absolute quantitation of total intact plasmalogens in future studies. Besides, only tuna meat was analyzed here, and the same investigation strategy could be extended to other food products containing plasmalogens at different concentrations and compositions. Since many disorders associated with metabolic syndrome (e.g., Alzheimer's disease) often have a long preclinical phase, diet could be more important in this phase than drugs for improving metabolic health. One associated strategy to this end is better preservation and control of functional components in foods, such as functional lipids represented by plasmalogens.

4. Conclusions

This study explored the decrease of plasmalogens and the simultaneous global changes of related lipids in ready-to-eat raw tuna meat during storage, including chilling, freezing, and thawing. Long-term cold storage caused a considerable loss of plasmalogens, and the storage duration and temperature were important factors. In particular, chilling under 4 °C for 15 days resulted in the loss of almost half of total plasmalogens. The plasmalogen fingerprints also showed characteristic changes during long-term storage. For the different headgroups, the ethanolamine type (i.e., PlsEtn) was less stable than the choline type (i.e., PlsCho). For the *sn*-2 fatty acyl chains, plasmalogen species containing PUFA were more likely to decrease

in concentration. In addition, although a single thawing step did not affect the plasmalogen level regardless of the employed thawing method, more than one freeze-thaw cycles led to extra loss of plasmalogens, especially PlsEtn. Regarding the possible mechanisms, the storage-induced plasmalogen loss might be attributed to oxidation and degradation. The accumulation of oxidized plasmalogens was strongly correlated with that of other lipid oxidation products and the degraded phospholipids, suggesting that plasmalogen loss could serve as a good indicator of meat quality deterioration. These findings not only provide additional information about the loss of functional phospholipids during food storage, but also indicate a more sensitive way to monitor the freshness and quality of ready-to-eat fish products.

Abbreviations

Artificial neural network (ANN), collision-induced dissociation (CID), diglycerides (DG), electrospray ionization (ESI), extracted ion chromatogram (EIC), free fatty acids (FFA), liquid chromatography (LC), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), malonaldehyde (MDA), mass spectrometry (MS), phosphatidylcholine (PC), phosphatidylethanolamine (PE), polyunsaturated fatty acyls (PUFA), principal component analysis (PCA), standard deviation (SD), 1,1,3,3-tetraethoxypropane (TEP), thiobarbituric acid (TBA), thiobarbituric acid-reactive substances (TBARS), triglycerides (TG).

Author contributions

Zhen Chen: Conceptualization, Funding acquisition, Investigation, Methodology, Visualization, Writing - original draft. **Jiaping Jia:** Data curation, Formal analysis, Investigation, Methodology, Resources. **Yue Wu:** Data curation, Formal analysis, Methodology, Software, Validation, Visualization, Writing - original draft. **Hitoshi Chiba:** Resources, Validation, review & editing. **Shu-Ping Hui:** Resources, Project administration, Supervision.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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592 **Figure captions**

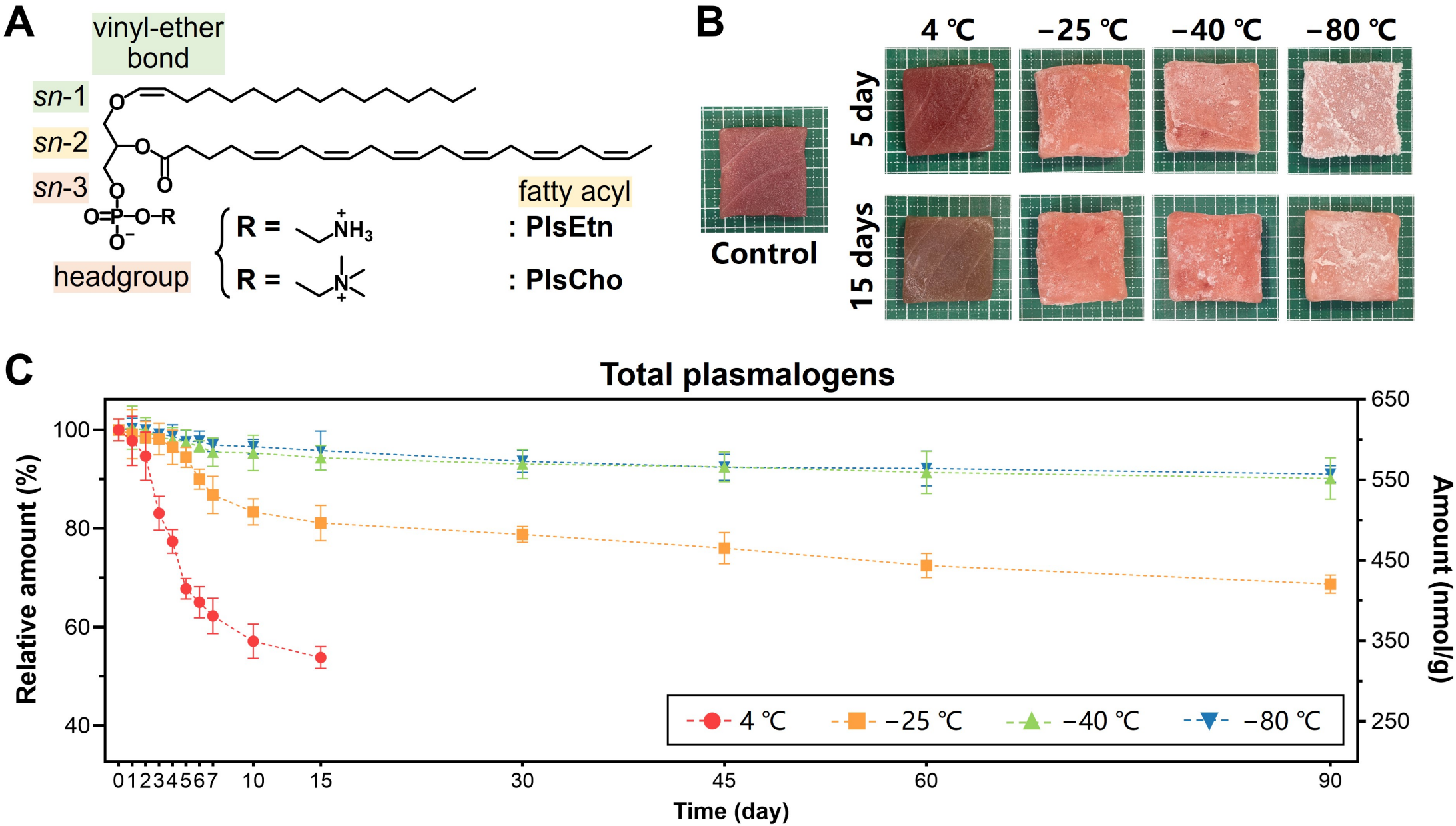
593 **Figure 1.** (A) Structure of plasmalogens consisting of a headgroup (ethanolamine or choline),
594 a fatty chain linked by vinyl-ether bond at the *sn*-1 position, and a fatty acyl chain at the *sn*-2
595 position. (B) Representative photographs of tuna lean meat samples on the 5^{th} and 15^{th} day of
596 storage under 4 °C, −25 °C, −40 °C, and −80 °C. (C) Changes in the levels of total
597 plasmalogens during long-term storage under 4 °C, −25 °C, −40 °C, and −80 °C.

598 **Figure 2.** (A) PCA score plots of plasmalogen profiles under different storage temperatures.
599 From left to right: −80 °C, −40 °C, −25 °C, and 4 °C. (B) Comparison of PlsEtn, PlsCho, PE,
600 and PC contents during long-term storage (up to 90 days under −25 °C, −40 °C, and −80 °C;
601 and up to 15 days under 4 °C). (C) Changes in fatty acyl content in PlsEtn and PlsCho during
602 long-term storage (up to 90 days under −25 °C, −40 °C, and −80 °C; and up to 15 days under
603 4 °C).

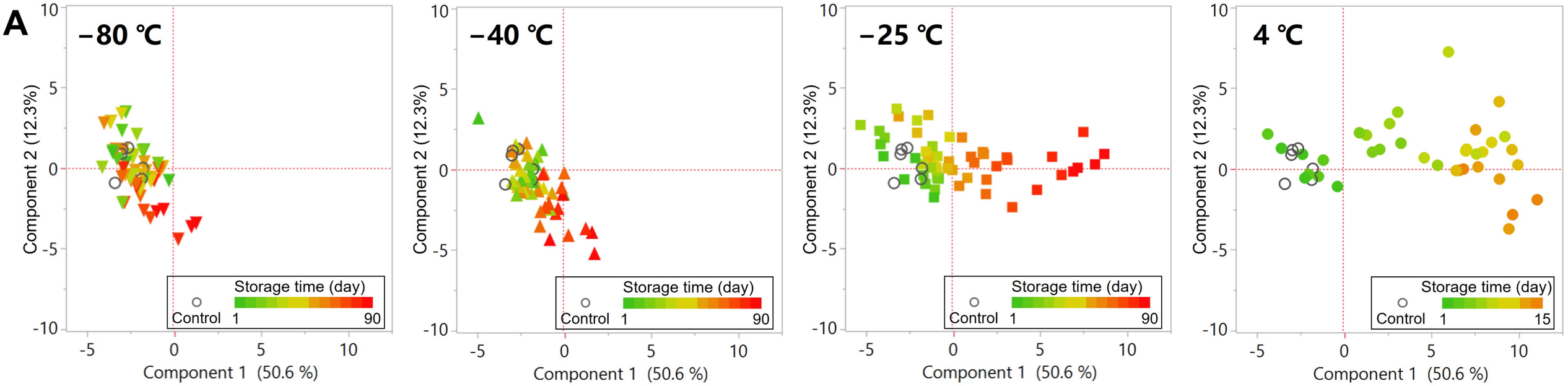
604 **Figure 3.** (A) Identification of oxidized plasmalogens by HR-MS and tandem MS, taking
605 PlsEtn 38:6+4[O] as an example. HR-MS provided the chemical formula of $C_{43}H_{74}O_{11}NP^-$,
606 indicating the existence of PlsEtn 38:6 + 4[O]. Further MS^2 and MS^3 analyses confirmed the
607 mixture of isomers PlsEtn p16:0(epo)/22:6(OH)(OOH) and PlsEtn p16:0$(OH)_2$(OOH). (B)
608 Accumulation of the oxidized PlsEtn and oxidized PlsCho (calculated as the sum of all
609 species) during storage under 4 °C, −25 °C, and −40 °C.

610 **Figure 4.** (A) Accumulation of lipid oxidation products (TG, DG, FFA, PC, and PE) during
611 storage under 4 °C, −25 °C, and −40 °C. (B) TBARS levels during storage under 4 °C,

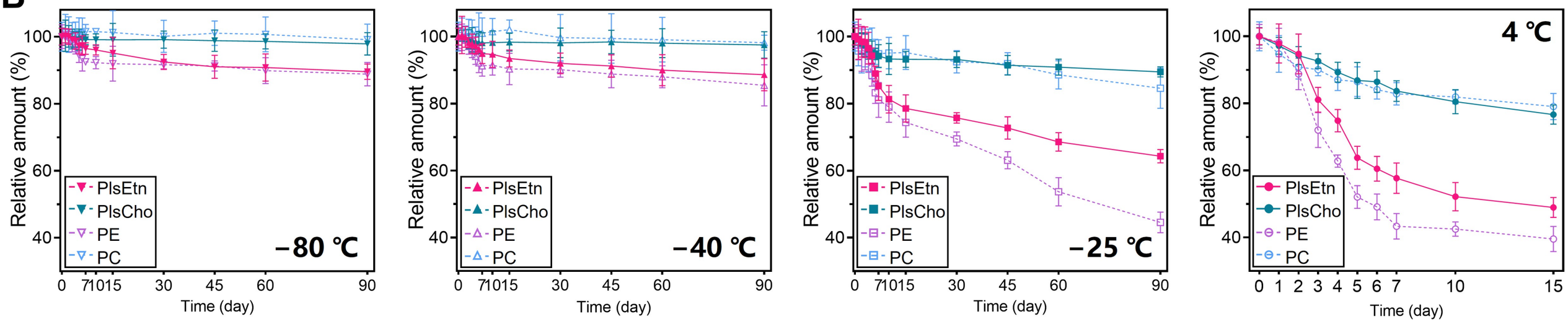
612 −25 °C, −40 °C, and −80 °C. (C) Increase in the levels of degraded phospholipids, namely
613 LPC, LPE, and FFA during storage under 4 °C, −25 °C, −40 °C, and −80 °C.
614 **Figure 5.** (A) Heatmap of the Spearman's correlation coefficients between the loss of
615 plasmalogens and the increase in the levels of related oxidized and degraded lipids. (B)
616 Scatter plots of TBARS level versus the loss rate of total plasmalogens under 4 °C, −25 °C,
617 −40 °C, and −80 °C. (C) Predictive three-dimensional surface plots of the remaining total
618 plasmalogens, PlsEtn, and PlsCho during long-term storage under different temperatures.
619 **Figure 6.** (A) Comparison of the contents of total plasmalogens, PlsEtn, and PlsCho in frozen
620 samples thawed using different methods. Con = control, WT = water immersion thawing,
621 MT = microwave thawing, RT = refrigerator thawing, AT = air thawing. (B) Comparison of
622 the contents of total plasmalogens, PlsEtn, and PlsCho during multiple freeze-thaw cycles. *,
623 $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, calculated using one-way ANOVA (using Tukey's
624 post hoc test).


A
vinyl-ether bond
sn-1
sn-2
sn-3
fatty acyl
headgroup
R = NH3 : PlsEtn
R = N+ : PlsCho
B
4 ℃
−25 ℃
−40 ℃
−80 ℃
5 day
15 days
Control
C
Total plasmalogens
Relative amount (%)
Amount (nmol/g)
Time (day)
4 ℃
−25 ℃
−40 ℃
−80 ℃

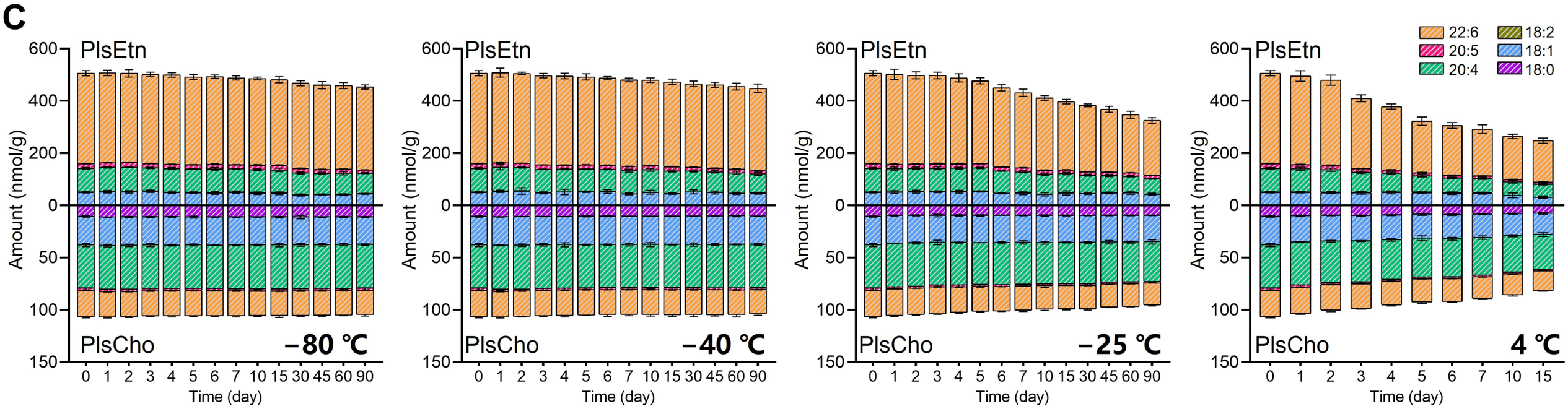
A

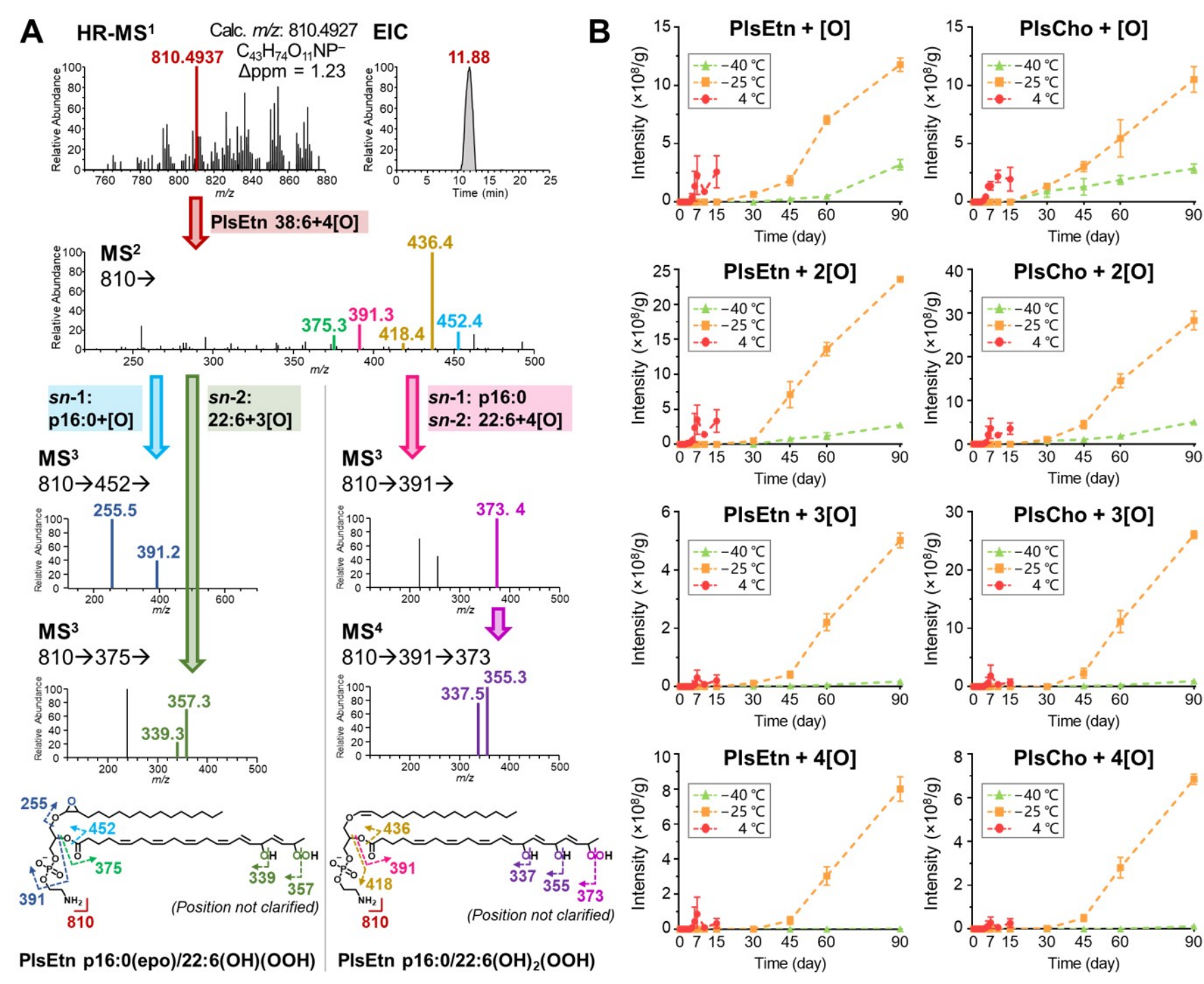

−80 ℃
−40 ℃
−25 ℃
4 ℃
Component 2 (12.3%)
Component 1 (50.6 %)
Storage time (day)
Control 1 90
Control 1 15

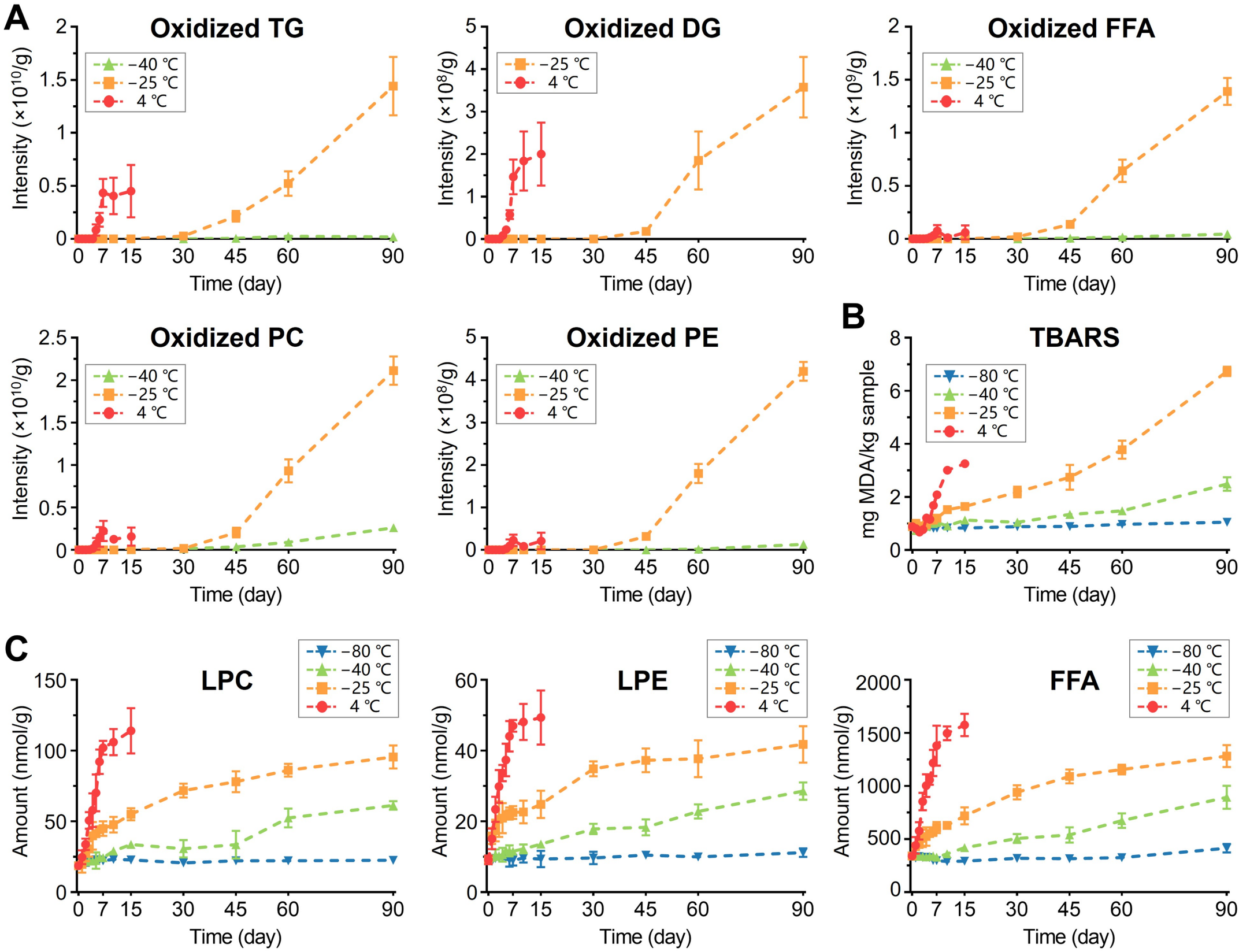
B


Relative amount (%)
PlsEtn
PlsCho
PE
PC
−80 ℃
−40 ℃
−25 ℃
4 ℃
Time (day)

C


PlsEtn
PlsCho
Amount (nmol/g)
22:6
20:5
20:4
18:2
18:1
18:0
−80 ℃
−40 ℃
−25 ℃
4 ℃
Time (day)


A
HR-MS¹
Calc. m/z: 810.4927
C43H74O11NP⁻
Δppm = 1.23
810.4937
EIC
11.88
Relative Abundance
m/z
Time (min)
PlsEtn 38:6+4[O]
MS²
810→
436.4
375.3
391.3
418.4
452.4
sn-1: p16:0+[O]
sn-2: 22:6+3[O]
sn-1: p16:0
sn-2: 22:6+4[O]
MS³
810→452→
255.5
391.2
MS³
810→391→
373. 4
MS³
810→375→
357.3
339.3
MS⁴
810→391→373
355.3
337.5
255
452
375
339
357
391
810
(Position not clarified)
436
391
418
337
355
373
810
(Position not clarified)
PlsEtn p16:0(epo)/22:6(OH)(OOH)
PlsEtn p16:0/22:6(OH)2(OOH)
B
PlsEtn + [O]
PlsCho + [O]
PlsEtn + 2[O]
PlsCho + 2[O]
PlsEtn + 3[O]
PlsCho + 3[O]
PlsEtn + 4[O]
PlsCho + 4[O]
Intensity (×10⁸/g)
Time (day)
−40 ℃
−25 ℃
4 ℃


A
Oxidized TG
Intensity (×10¹⁰/g)
−40 ℃
−25 ℃
4 ℃
Time (day)
Oxidized DG
Intensity (×10⁸/g)
−25 ℃
4 ℃
Time (day)
Oxidized FFA
Intensity (×10⁹/g)
−40 ℃
−25 ℃
4 ℃
Time (day)
Oxidized PC
Intensity (×10¹⁰/g)
−40 ℃
−25 ℃
4 ℃
Time (day)
Oxidized PE
Intensity (×10⁸/g)
−40 ℃
−25 ℃
4 ℃
Time (day)
B
TBARS
mg MDA/kg sample
−80 ℃
−40 ℃
−25 ℃
4 ℃
Time (day)
C
LPC
Amount (nmol/g)
−80 ℃
−40 ℃
−25 ℃
4 ℃
Time (day)
LPE
Amount (nmol/g)
−80 ℃
−40 ℃
−25 ℃
4 ℃
Time (day)
FFA
Amount (nmol/g)
−80 ℃
−40 ℃
−25 ℃
4 ℃
Time (day)

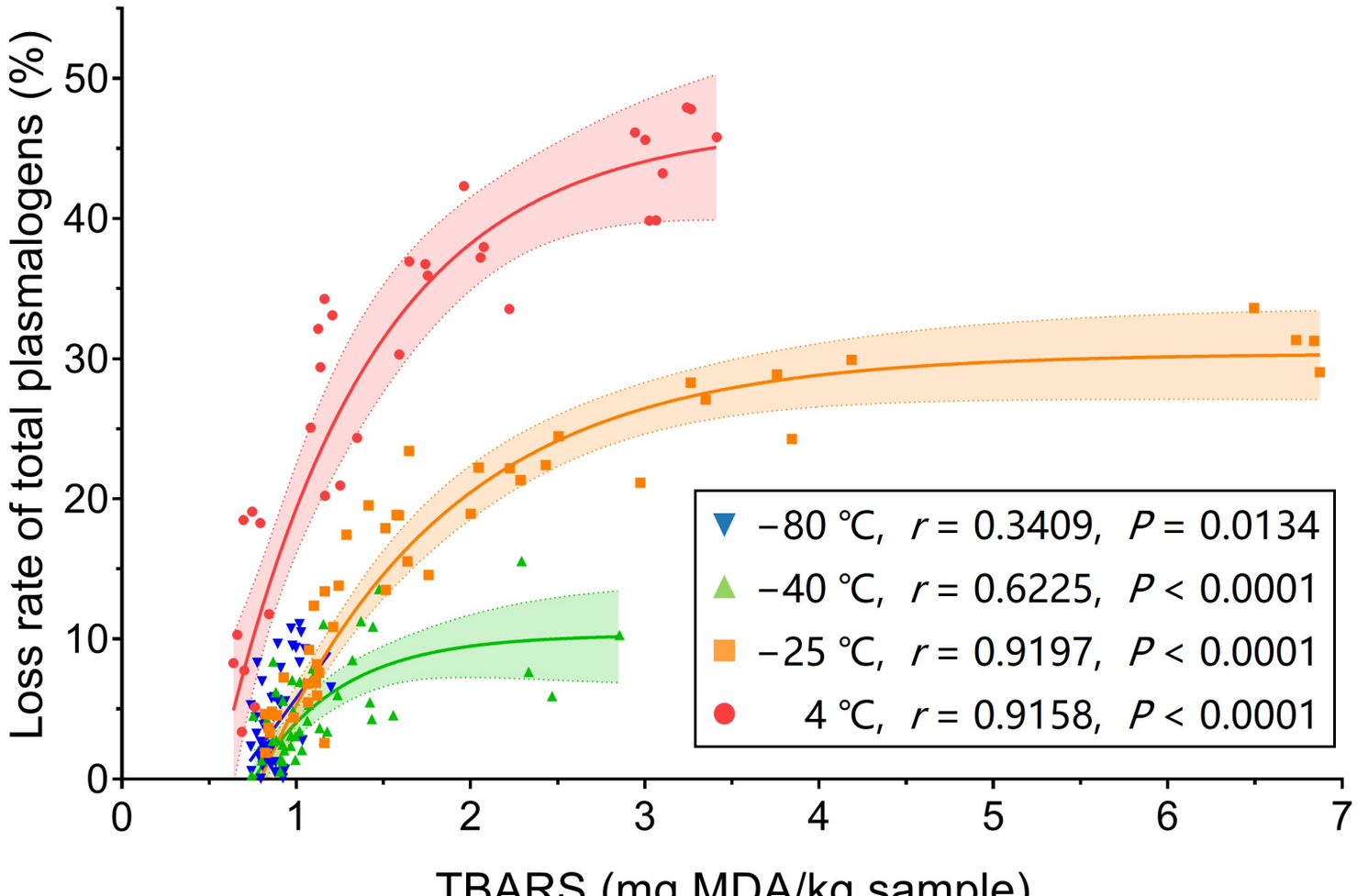
A

Total	PlsEtn	PlsCho	Total	PlsEtn	PlsCho	
0.86	0.86	0.73	0.77	0.77	0.58	PlsEtn+1[O]
0.94	0.93	0.85	0.77	0.77	0.59	PlsEtn+2[O]
0.85	0.84	0.70	0.77	0.77	0.58	PlsEtn+3[O]
0.84	0.84	0.70	0.69	0.69	0.56	PlsEtn+4[O]
0.92	0.92	0.83	0.77	0.77	0.59	PlsCho+1[O]
0.93	0.93	0.82	0.77	0.77	0.58	PlsCho+2[O]
0.87	0.87	0.73	0.69	0.69	0.56	PlsCho+3[O]
0.88	0.88	0.75	0.77	0.77	0.59	PlsCho+4[O]
0.89	0.89	0.81	0.77	0.77	0.58	oxTG
0.94	0.93	0.88	0.69	0.69	0.56	oxDG
0.90	0.90	0.80	0.77	0.77	0.59	oxFFA
0.92	0.92	0.81	0.92	0.91	0.69	oxPC
0.85	0.84	0.71	0.69	0.69	0.56	oxPE
0.85	0.85	0.80	0.89	0.88	0.79	TBARS
0.95	0.94	0.87	0.92	0.91	0.65	LPC
0.90	0.90	0.85	0.79	0.78	0.76	LPE
0.93	0.92	0.90	0.86	0.85	0.80	FFA

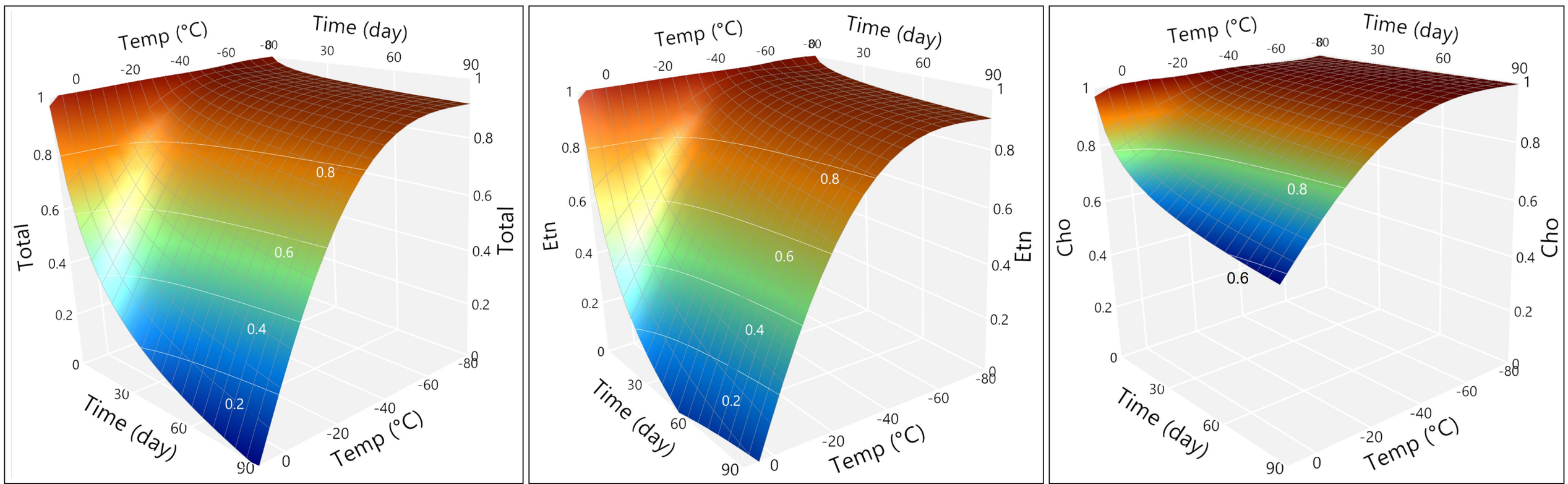
Loss at 4 °C | Loss at −25 °C

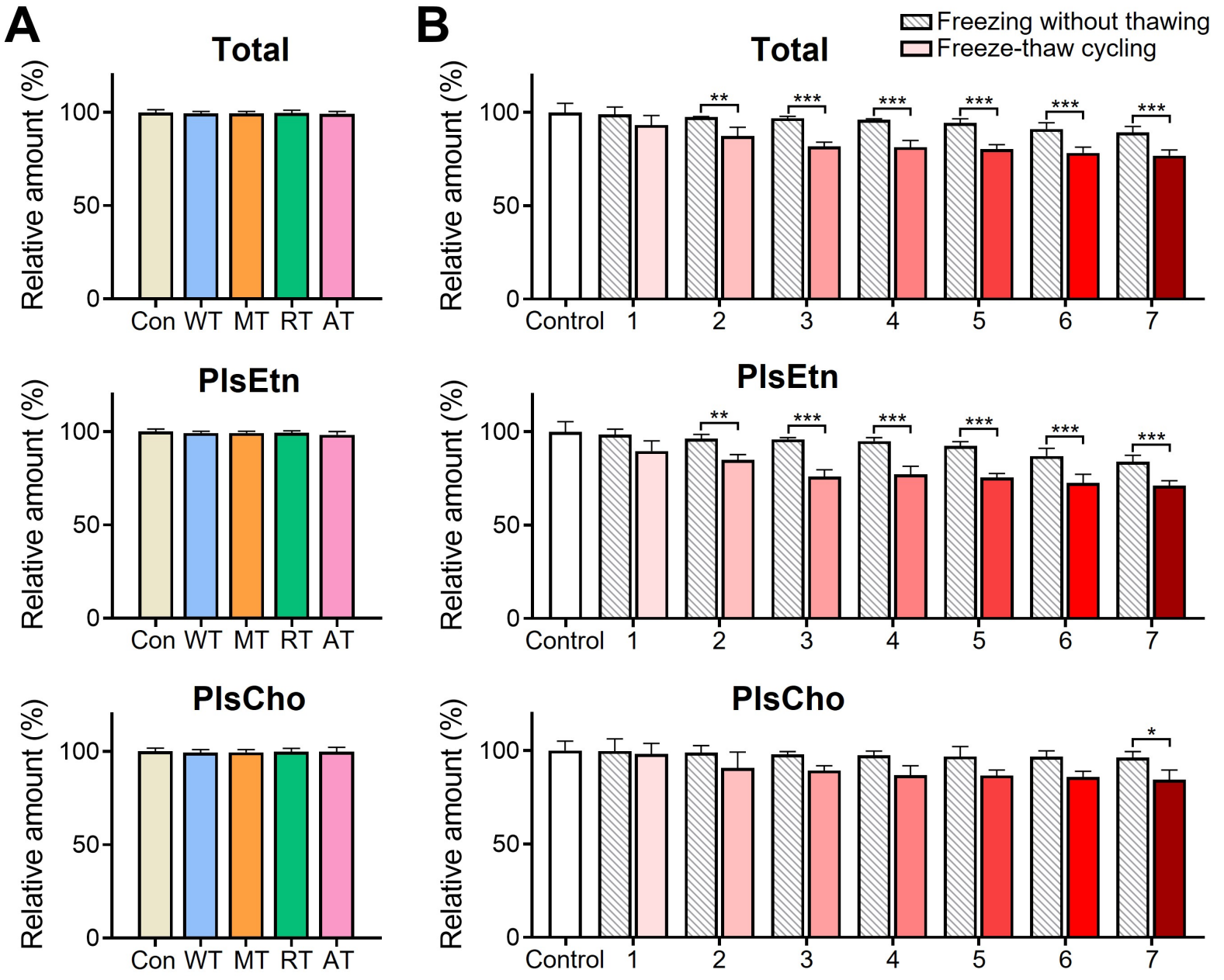
1 0.9 0.8 0.7 0.6 0.5

B



C




A
Total
PlsEtn
PlsCho
Relative amount (%)
100
50
0
Con WT MT RT AT
B
Freezing without thawing
Freeze-thaw cycling
Total
PlsEtn
PlsCho
Relative amount (%)
Control 1 2 3 4 5 6 7
**
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*

Supplementary

Table S1. Information of each lipid internal standard

Standard	Source	Concentration in MeOH stock solution (nmol/mL)	Amount in each sample (nmol/per tube)
Choline plasmalogen p16:0/17:0 (PlsCho p16:0/17:0)	Laboratory	15.0	1.5
Ethanolamine plasmalogen p16:0/17:0 (PlsEtn p16:0/17:0)	Laboratory	10.0	1.0
Phosphatidylcholine (PC 13:0/13:0)	Avanti Polar Lipids	15.0	1.5
Phosphatidylethanolamine (PE 15:0/15:0)	Avanti Polar Lipids	10.0	1.0
Lysophosphatidylcholine (LPC 15:0)	Avanti Polar Lipids	15.0	1.5
Lysophosphatidylethanolamine (LPE 13:0)	Avanti Polar Lipids	10.0	1.0
Free fatty acid (FFA 17:0)	Sigma-Aldrich	10.0	1.0

Table S2. Identification of intact plasmalogens in fish samples

Lipid species	Retention time (min)	Ion	Calc. *m/z*	Exp. *m/z*	Δppm	MS/MS signals	Molecule species
PlsEtn							
PlsEtn32:1	15.48	$[M-H]^-$	672.4974	672.4987	-1.93	281,408,390 253,436,418	p14:0/18:1 p16:0/16:1
PlsEtn34:1	16.39	$[M-H]^-$	700.5287	700.5287	0.00	281,436,418	p16:0/18:1
PlsEtn34:2	15.85	$[M-H]^-$	698.5130	698.5127	0.43	279,436,418 281,434,416	p16:0/18:2 p16:1/18:1
PlsEtn34:4	14.92	$[M-H]^-$	694.4817	694.4829	-1.73	303,408,390	p14:0/20:4
PlsEtn34:5	14.47	$[M-H]^-$	692.4661	692.4673	-1.73	301,408,390	p14:0/20:5
PlsEtn36:1	17.17	$[M-H]^-$	728.5600	728.5584	2.20	281,464,446	p18:0/18:1
PlsEtn36:2	16.51	$[M-H]^-$	726.5443	726.5448	-0.69	281,462,444	p18:1/18:1
PlsEtn36:4	15.73	$[M-H]^-$	722.5130	722.5150	-2.77	303,436,418	p16:0/20:4
PlsEtn36:5	15.29	$[M-H]^-$	720.4974	720.4991	-2.36	301,436,418	p16:0/20:5
PlsEtn36:6	14.80	$[M-H]^-$	718.4817	718.4825	-1.11	327,408,390	p14:0/22:6
PlsEtn38:4	16.58	$[M-H]^-$	750.5443	750.5442	0.13	303,464,446	p18:0/20:4
PlsEtn38:5	16.07	$[M-H]^-$	748.5287	748.5301	-1.87	303,462,444 329,436,418 301,464,446	p18:1/20:4 p16:0/22:5 p18:0/20:5
PlsEtn38:6	15.66	$[M-H]^-$	746.5130	746.5146	-2.14	327,436,418	p16:0/22:6
PlsEtn38:7	14.95	$[M-H]^-$	744.4974	744.4989	-2.01	327,434,416 301,460,442	p16:1/22:6 p18:2/20:5
PlsEtn38:8	14.43	$[M-H]^-$	742.4817	742.4819	-0.27	327,432,414	p16:2/22:6
PlsEtn40:6	16.45	$[M-H]^-$	774.5443	774.5457	-1.81	327,464,446	p18:0/22:6
PlsEtn40:7	15.79	$[M-H]^-$	772.5287	772.5292	-0.65	327,462,444	p18:1/22:6
PlsEtn40:8	15.29	$[M-H]^-$	770.5130	770.5132	-0.26	327,460,442	p18:2/22:6
PlsCho							
PlsCho34:0	18.70	$[M+CH_3COO]^-$	804.6124	804.6125	-0.12	730,283,464	p16:0/18:0
PlsCho34:1	17.97	$[M+CH_3COO]^-$	802.5967	802.5984	-2.12	728,281,464	p16:0/18:1
PlsCho36:4	16.10	$[M+CH_3COO]^-$	824.5811	824.5811	0.00	750,303,464	p16:0/20:4
PlsCho36:5	15.10	$[M+CH_3COO]^-$	822.5654	822.5668	-1.70	748,301,464	p16:0/20:5
PlsCho38:4	17.76	$[M+CH_3COO]^-$	852.6124	852.6118	0.70	778,303,492	p18:0/20:4
PlsCho38:5	16.26	$[M+CH_3COO]^-$	850.5967	850.5972	-0.59	776,303,490	p18:1/20:4
PlsCho38:6	15.82	$[M+CH_3COO]^-$	848.5811	848.5824	-1.53	774,327,464	p16:0/22:6
PlsCho40:6	17.62	$[M+CH_3COO]^-$	876.6124	876.6124	0.00	802,327,492	p18:0/22:6

Table S3. Identification of oxidized plasmalogens in fish samples

Lipid species	Retention time (min)	Ion	Calc. *m/z*	Exp *m/z*	Δppm
PlsEtn+1[O]					
PlsEtn36:4+1[O]	14.13	$[M-H]^-$	738.5079	738.5084	-0.68
PlsEtn38:4+1[O]	14.91	$[M-H]^-$	766.5392	766.5388	0.52
PlsEtn38:5+1[O]	14.49	$[M-H]^-$	764.5236	764.5250	-1.83
PlsEtn38:6+1[O]	13.95	$[M-H]^-$	762.5079	762.5082	-0.39
PlsEtn40:6+1[O]	14.67	$[M-H]^-$	790.5392	790.5394	-0.25
PlsEtn40:7+1[O]	14.10	$[M-H]^-$	788.5236	788.5234	0.25
PlsEtn40:8+1[O]	14.04	$[M-H]^-$	786.5079	786.5079	0.00
PlsCho+1[O]					
PlsCho38:5+1[O]	14.97	$[M+CH_3COO]^-$	866.5917	866.5913	0.46
PlsCho38:6+1[O]	14.22	$[M+CH_3COO]^-$	864.5760	864.5763	-0.35
PlsEtn+2[O]					
PlsEtn36:4+2[O]	13.83	$[M-H]^-$	754.5028	754.5023	0.66
PlsEtn38:4+2[O]	14.46	$[M-H]^-$	782.5341	782.5346	-0.64
PlsEtn38:5+2[O]	13.95	$[M-H]^-$	780.5185	780.5181	0.51
PlsEtn38:6+2[O]	13.56	$[M-H]^-$	778.5028	778.5028	0.00
PlsEtn40:6+2[O]	14.58	$[M-H]^-$	806.5341	806.5345	-0.50
PlsEtn40:7+2[O]	13.62	$[M-H]^-$	804.5185	804.5189	-0.50
PlsCho+2[O]					
PlsCho36:4+2[O]	14.25	$[M+CH_3COO]^-$	856.5709	856.5715	-0.70
PlsCho36:5+2[O]	13.43	$[M+CH_3COO]^-$	854.5553	854.5532	2.46
PlsCho38:5+2[O]	14.25	$[M+CH_3COO]^-$	882.5866	882.5860	0.68
PlsCho38:6+2[O]	13.77	$[M+CH_3COO]^-$	880.5709	880.5718	-1.02
PlsCho40:6+2[O]	15.64	$[M+CH_3COO]^-$	908.6022	908.6025	-0.33
PlsEtn+3[O]					
PlsEtn38:4+3[O]	13.26	$[M-H]^-$	798.5291	798.5295	-0.50
PlsEtn38:5+3[O]	12.41	$[M-H]^-$	796.5134	796.5143	-1.13
PlsEtn38:6+3[O]	12.21	$[M-H]^-$	794.4978	794.4985	-0.88
PlsEtn38:7+3[O]	12.08	$[M-H]^-$	792.4821	792.4830	-1.14
PlsEtn40:6+3[O]	12.68	$[M-H]^-$	822.5291	822.5297	-0.73
PlsEtn40:7+3[O]	12.35	$[M-H]^-$	820.5134	820.5151	-2.07
PlsEtn40:8+3[O]	12.24	$[M-H]^-$	818.4978	818.4980	-0.24

Lipid species	Retention time (min)	Ion	Calc. *m/z*	Exp *m/z*	Δppm
PlsCho+3[O]					
PlsCho36:4+3[O]	13.59	$[M+CH_3COO]^-$	872.5658	872.5653	0.57
PlsCho36:5+3[O]	13.20	$[M+CH_3COO]^-$	870.5502	870.5505	-0.34
PlsCho38:5+3[O]	14.13	$[M+CH_3COO]^-$	898.5815	898.5814	0.11
PlsCho38:6+3[O]	13.46	$[M+CH_3COO]^-$	896.5658	896.5662	-0.45
PlsCho40:6+3[O]	14.55	$[M+CH_3COO]^-$	924.5971	924.5977	-0.65
PlsEtn+4[O]					
PlsEtn36:4+4[O]	12.02	$[M-H]^-$	786.4927	786.4907	2.54
PlsEtn38:5+4[O]	12.02	$[M-H]^-$	812.5083	812.5085	-0.25
PlsEtn38:6+4[O]	11.88	$[M-H]^-$	810.4927	810.4937	-1.23
PlsEtn38:7+4[O]	11.64	$[M-H]^-$	808.4770	808.4782	-1.48
PlsEtn40:6+4[O]	12.18	$[M-H]^-$	838.5240	838.5257	-2.03
PlsCho+4[O]					
PlsCho38:5+4[O]	13.32	$[M+CH_3COO]^-$	914.5764	914.5787	-2.51
PlsCho38:6+4[O]	12.71	$[M+CH_3COO]^-$	912.5608	912.5616	-0.88
PlsCho40:6+4[O]	13.59	$[M+CH_3COO]^-$	940.5921	940.5923	-0.21

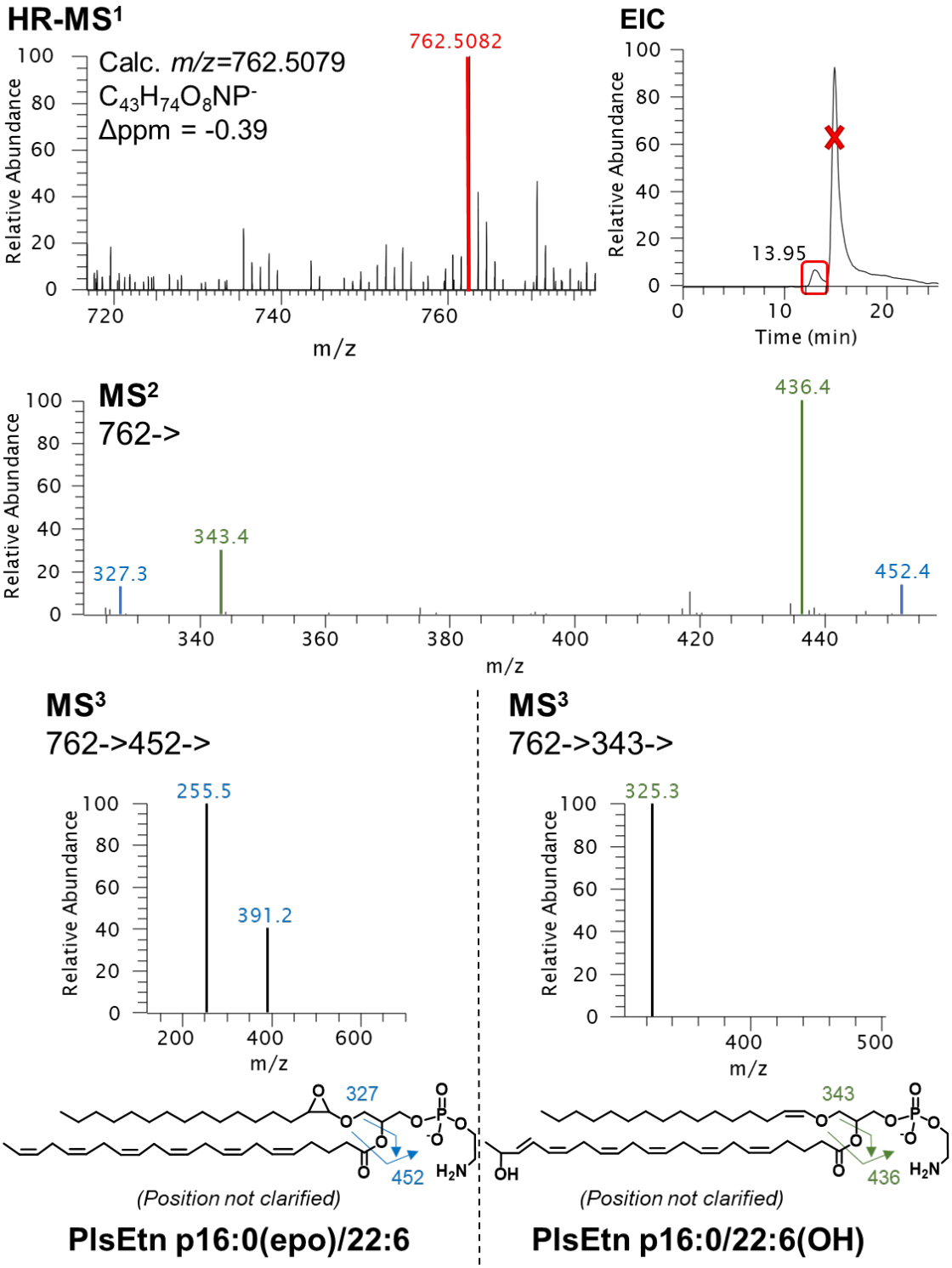


Figure S1. Identification of PlsEtn 38:6+1[O] by HR-MS and tandem MS.

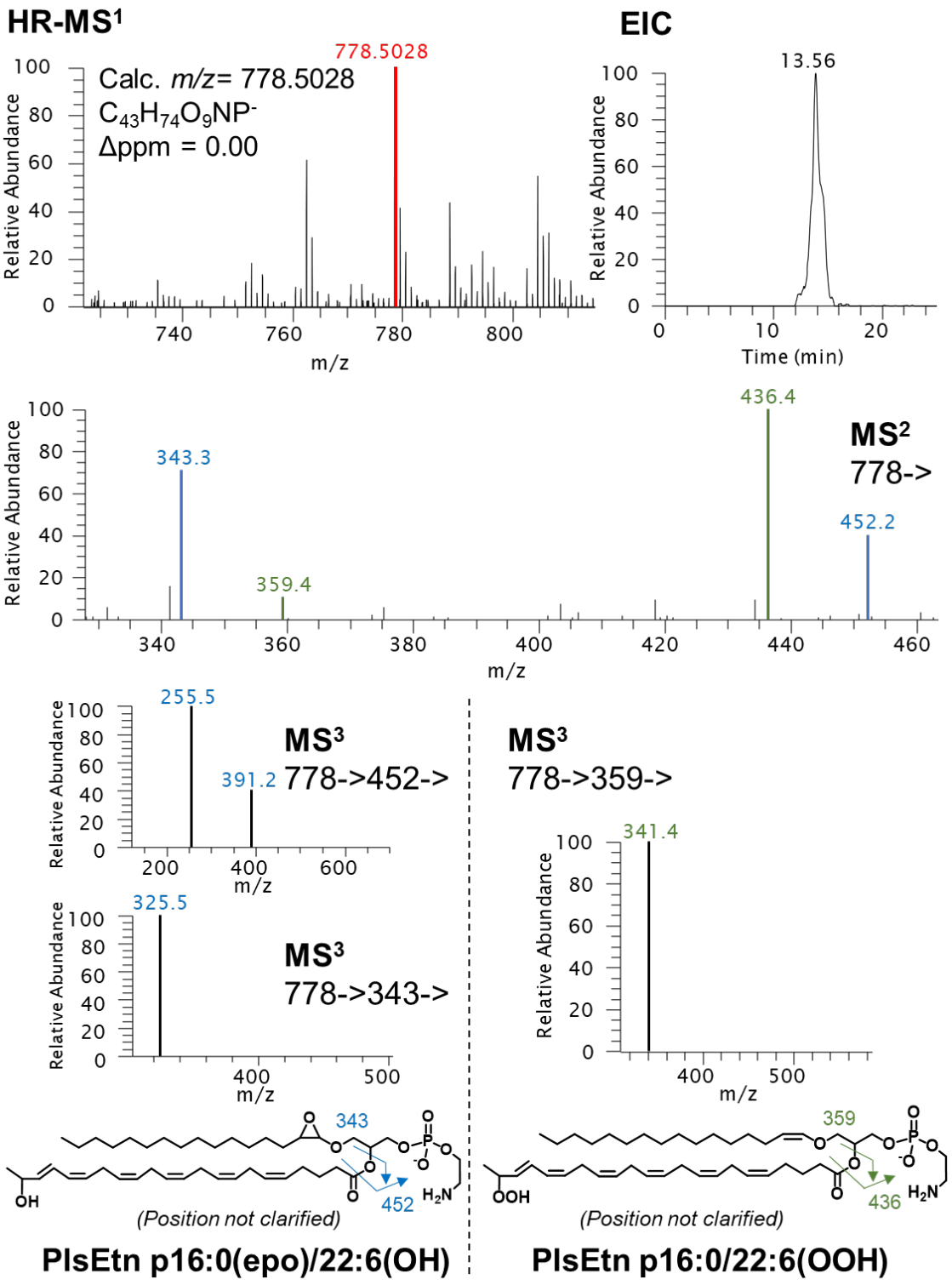


Figure S2. Identification of PlsEtn 38:6+2[O] by HR-MS and tandem MS.

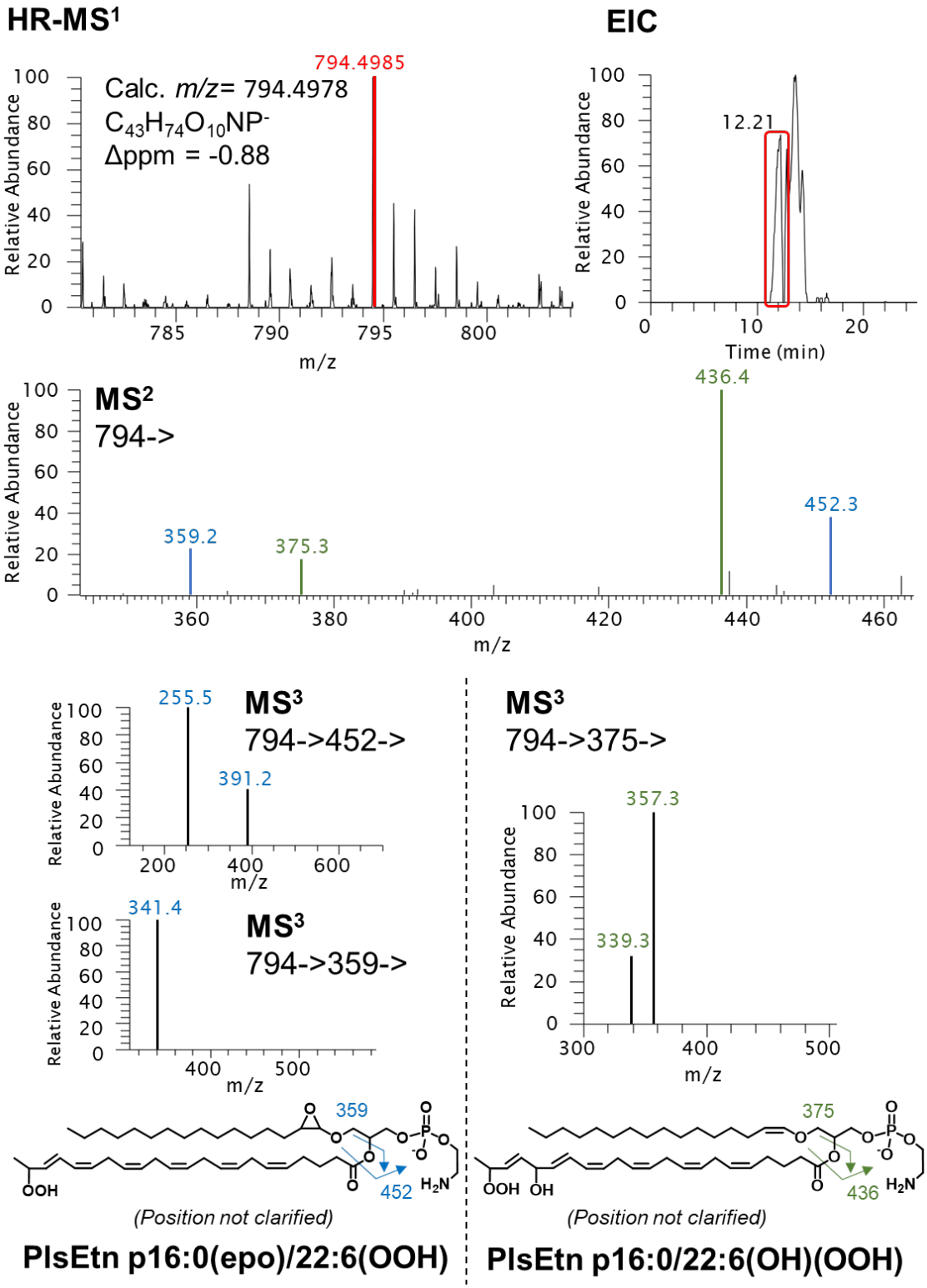


Figure S3. Identification of PlsEtn 38:6+3[O] by HR-MS and tandem MS.

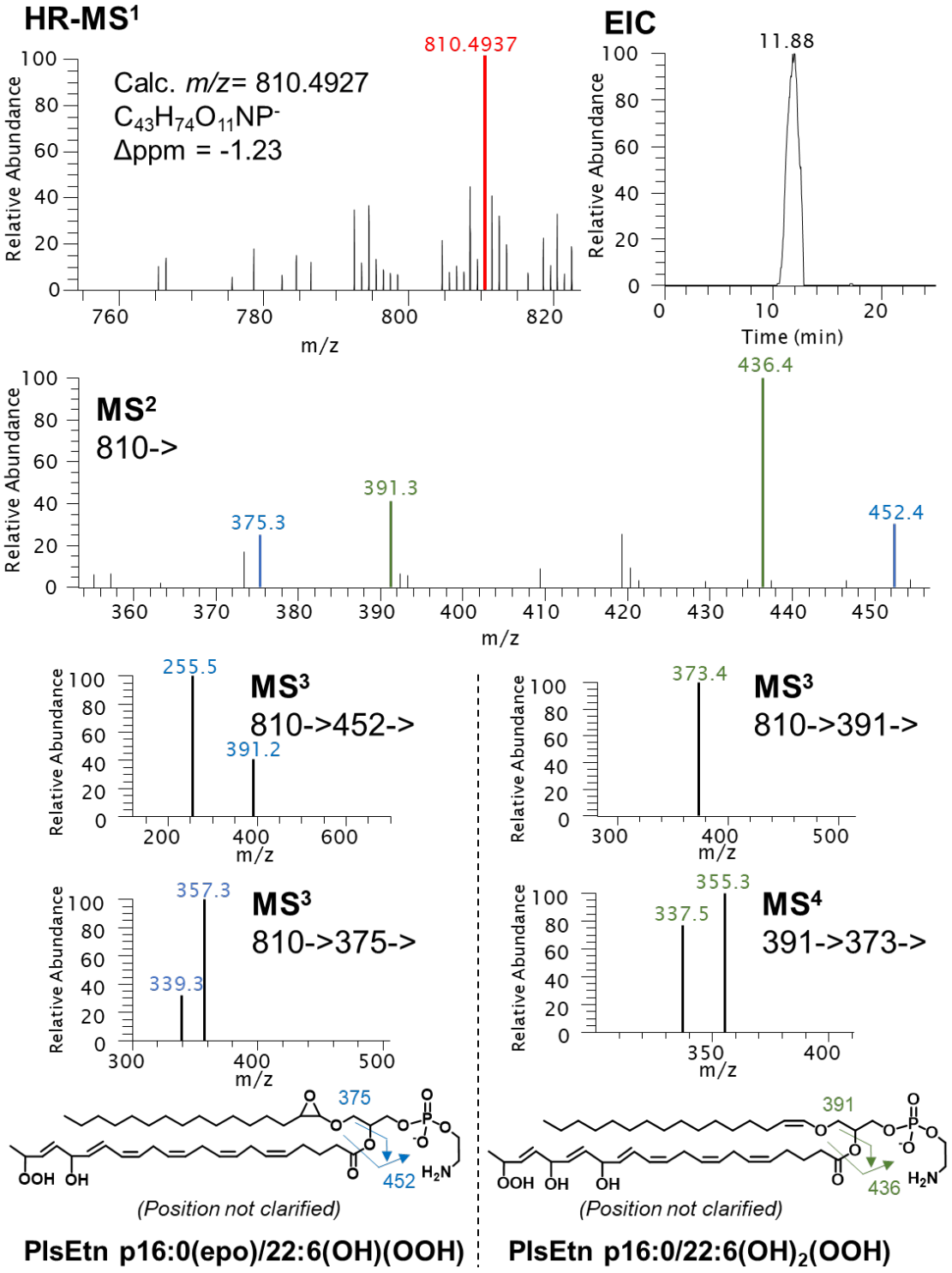


Figure S4. Identification of PlsEtn 38:6+4[O] by HR-MS and tandem MS.

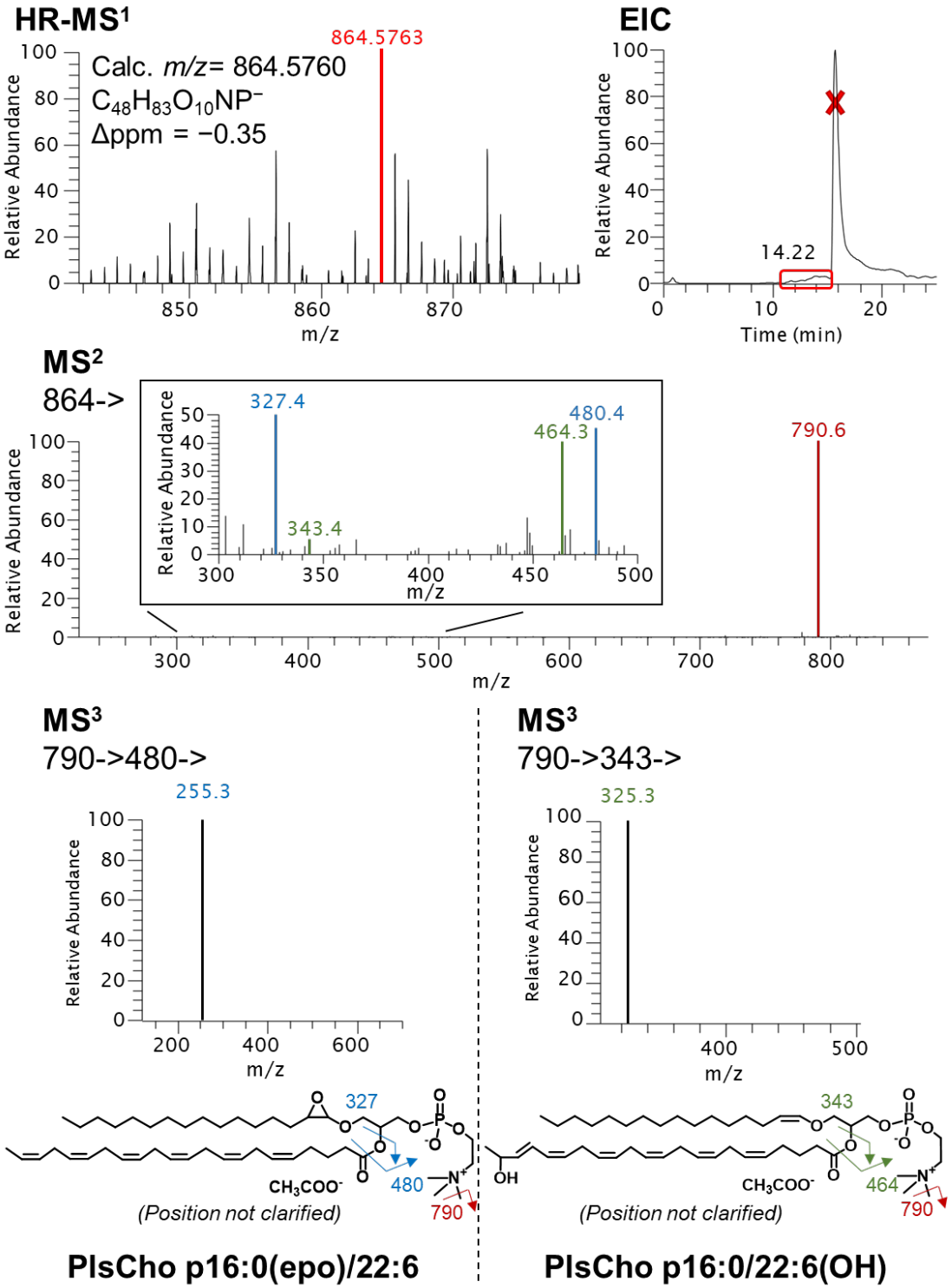


Figure S5. Identification of PlsCho 38:6+1[O] by HR-MS and tandem MS.

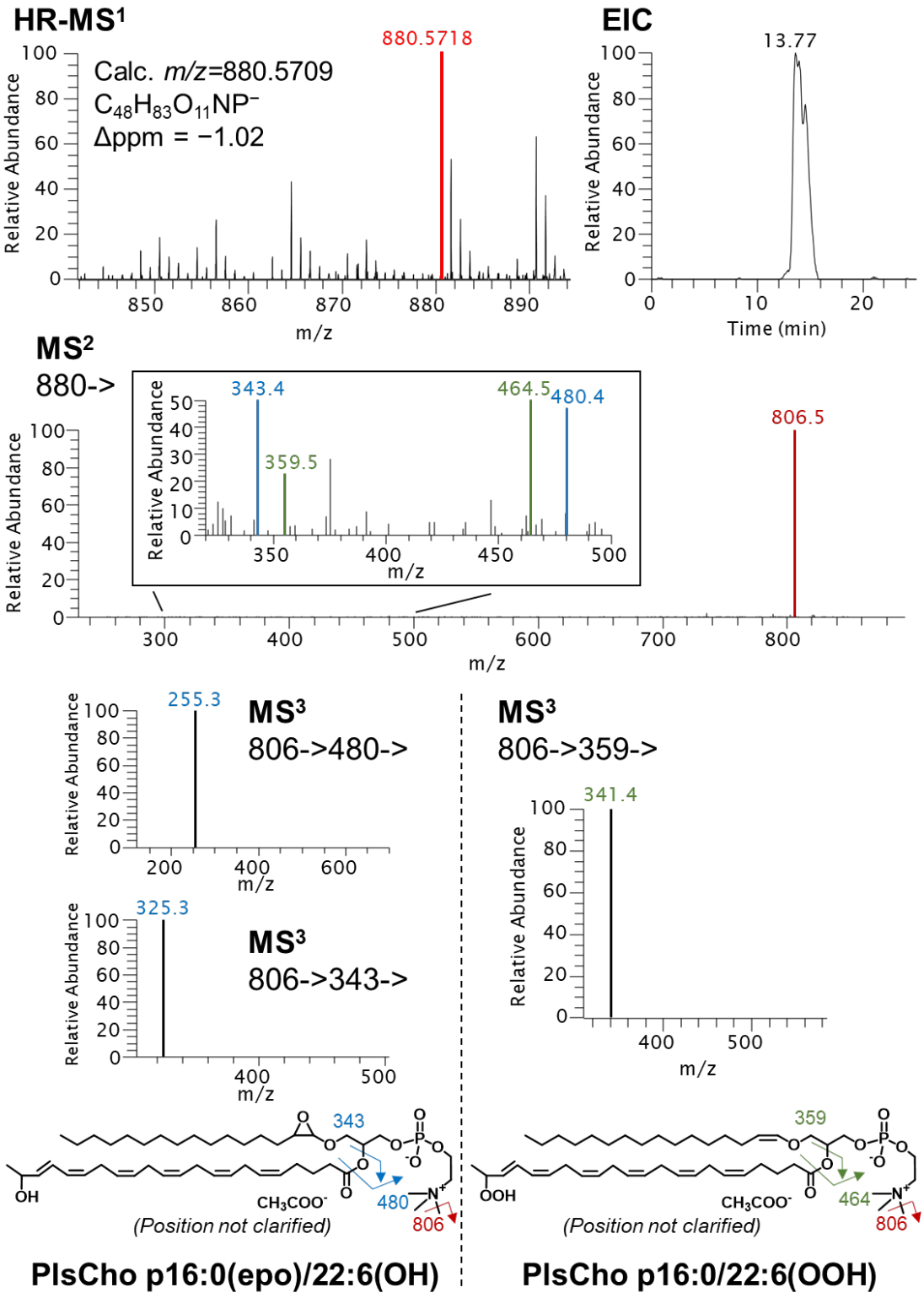


Figure S6. Identification of PlsCho 38:6+2[O] by HR-MS and tandem MS.

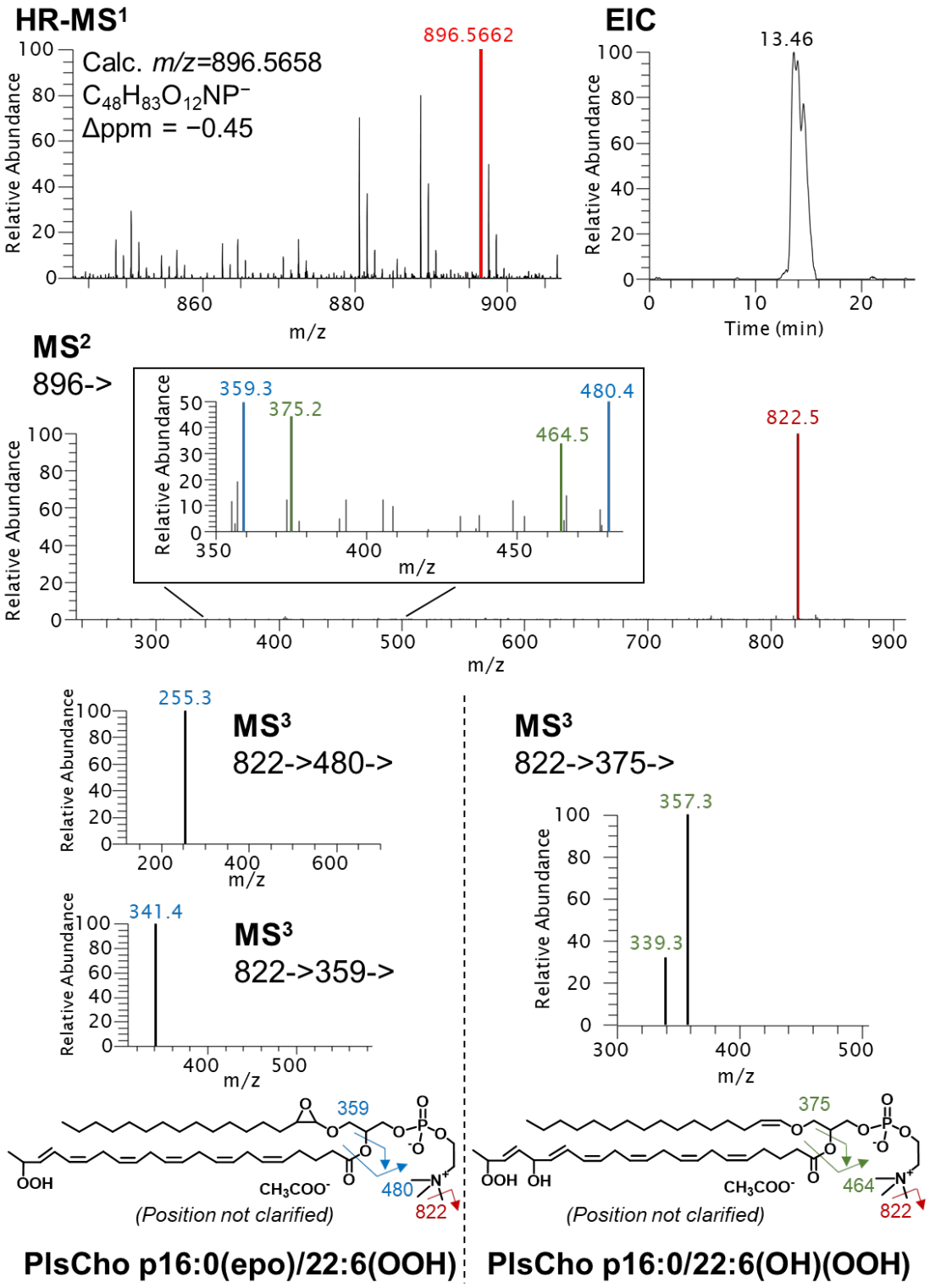


Figure S7. Identification of PlsCho 38:6+3[O] by HR-MS and tandem MS.

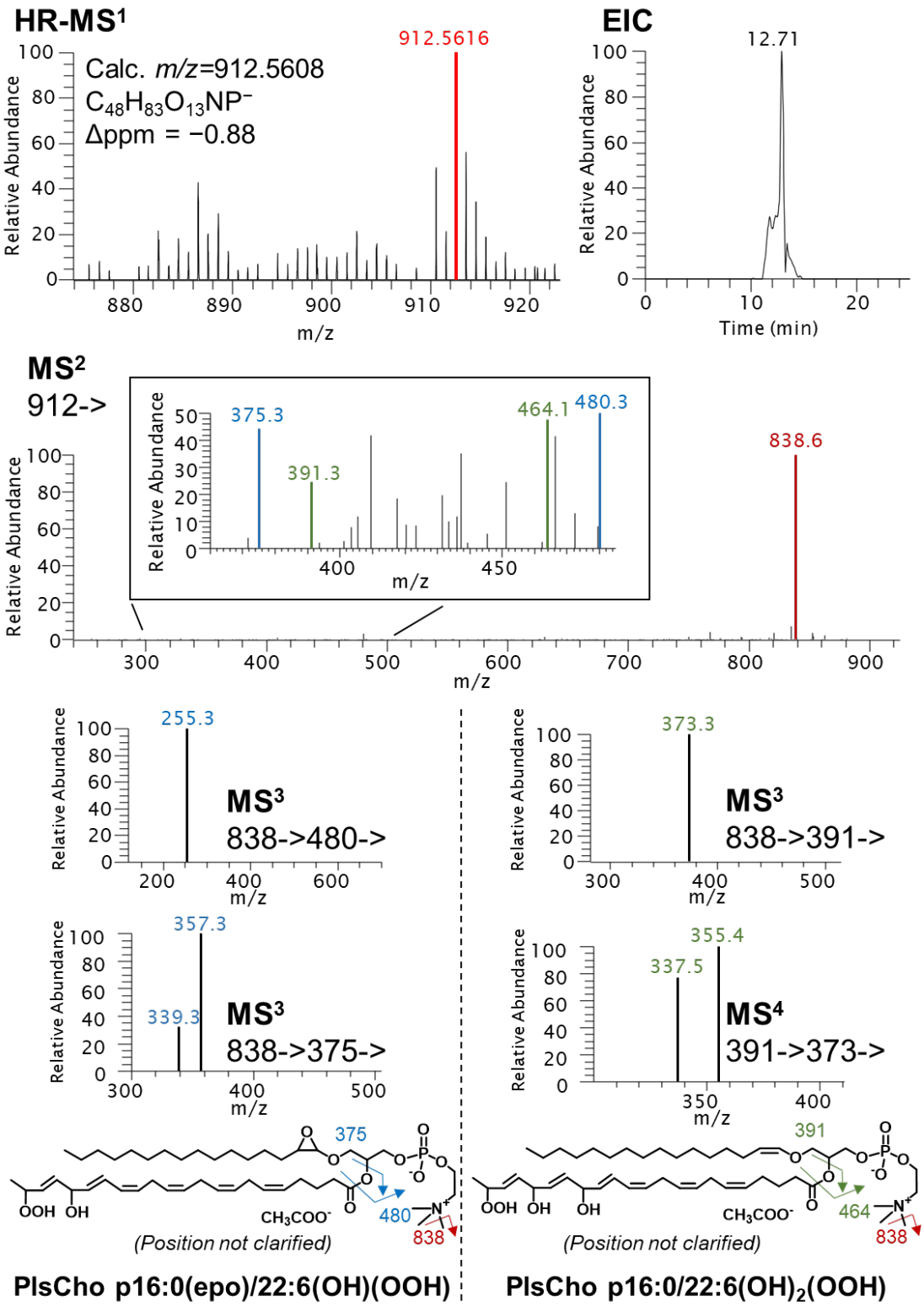


Figure S8. Identification of PlsCho 38:6+4[O] by HR-MS and tandem MS.

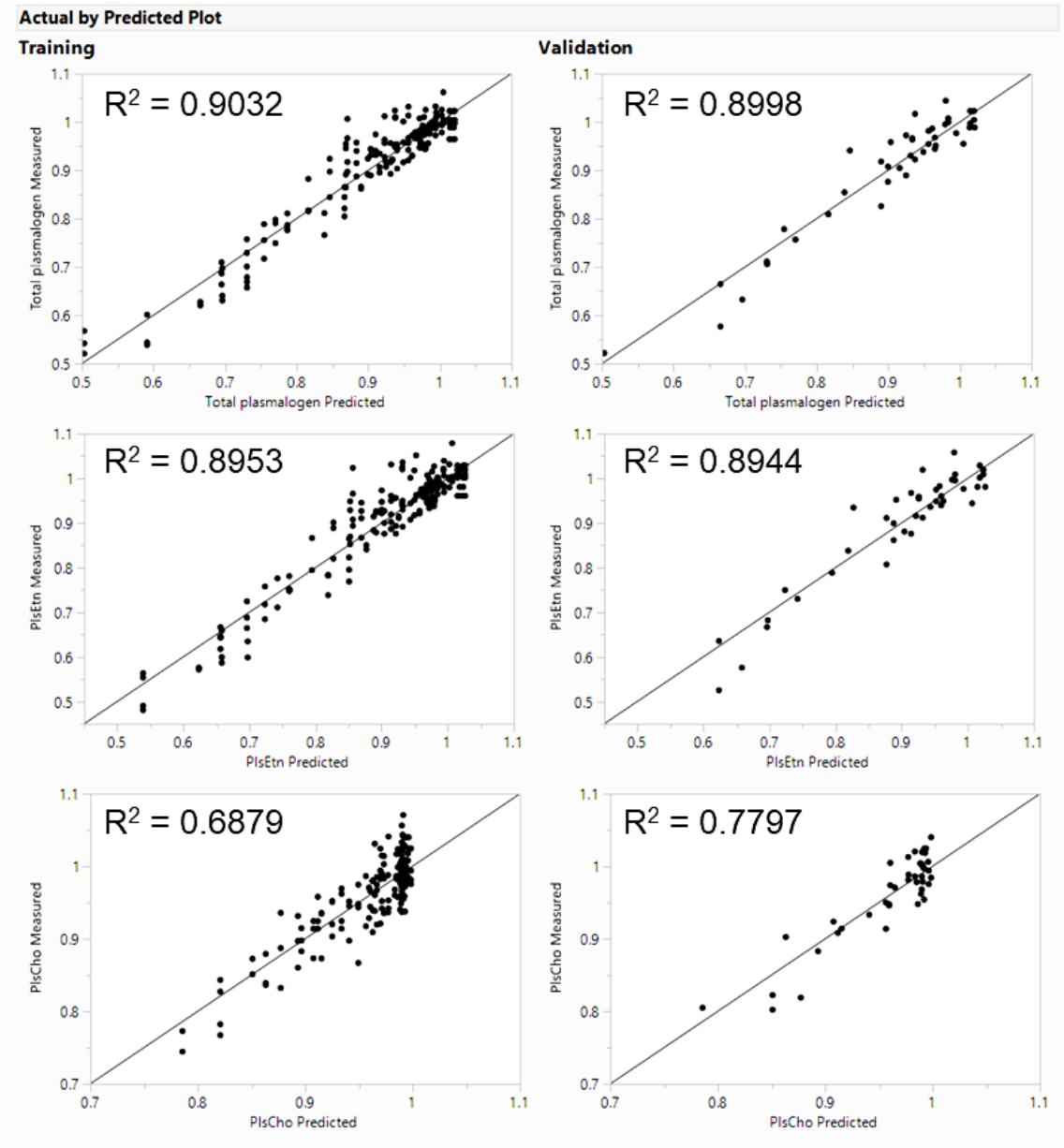
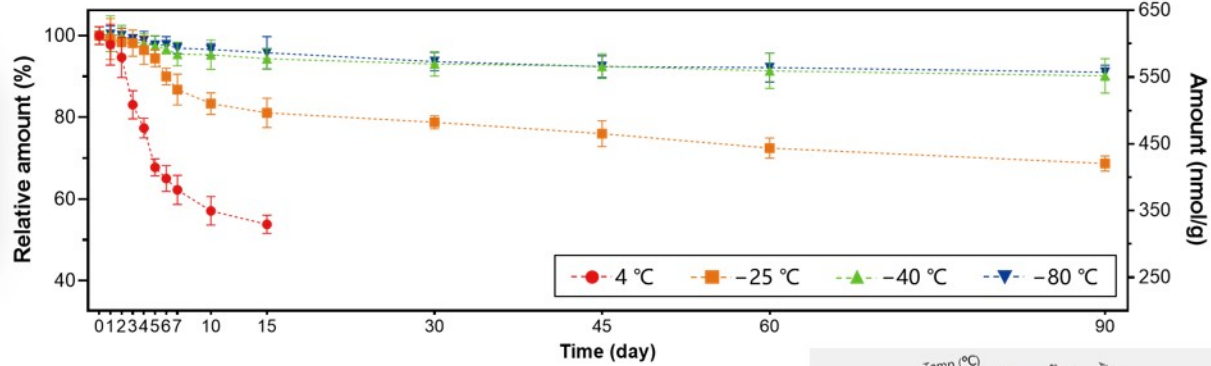
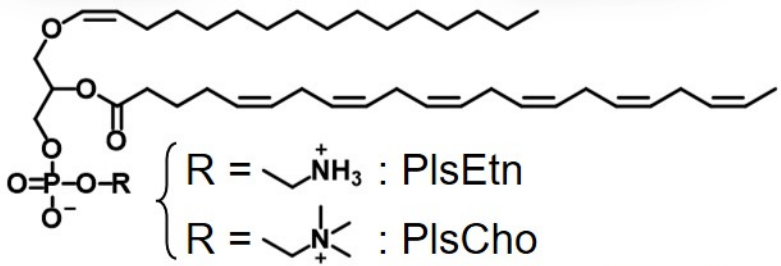


Figure S9. Measured vs. predicted values in training and validation data set for the remained content of the plasmalogen species during storage.

Chilling
Freezing





Plasmalogens → Oxidation
Plasmalogens → Degradation