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Research Article

Improved assay for malondialdehyde determination in surimi by gas chromatography-mass spectrometry after alkaline hydrolysis

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Abstract: Malondialdehyde (MDA) content is a primary indicator evaluating lipid oxidation level in oil-containing foods. However, total MDA compounds (tMDA) exist in both free MDA (fMDA) and bound MDA (bMDA) forms at the stage of lipid peroxidation. The traditional spectrophotometric assay quantifies the MDA after an acid hydrolysis process, only releasing limited levels of fMDA from bMDA, which named as rMDA. Considering lack of study about the effect of hydrolysis treatments on the efficiency and result accuracy in terms of MDA detection for fish products. In this study, an alkaline hydrolysis method was compared with the conventional trichloroacetic acid (TCA) hydrolysis. The results showed that the highest MDA content was obtained under hydrolysis of surimi samples with 1 mol/L NaOH for 60 min at 60 °C, which was about 2 times of that obtained from 15 g/100 mL TCA for 30 min. The alkaline hydrolysis was proved to produce more rMDA, exhibiting higher hydrolysis efficiency over the conventional method. Present works also optimized a protocol on derivatization and liquid-liquid extraction process prior to gas chromatography-mass spectrometry quantification. The hydrolysate was firstly regulated by HCl, allowing protein to fully precipitated by the following addition of TCA. This developed liquid-liquid extraction could reduce interference from presence of protein in the aqueous phase, achieving a more sensitive result with improved precision and recovery.

Keywords: malondialdehyde; gas chromatography-mass spectrometry; lipid peroxidation; hydrolysis efficiency; acetonitrile extraction; analytical protocol

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1 Introduction

Lipid oxidation could form various oxidative products in foods. Malondialdehyde (MDA) content is a representative indicator produced from lipid peroxidation stage of polyunsaturated fatty acids (PUFA)[1]. Therefore, PUFA-rich foods such as fatty fish and processed meat products depend on the MDA assay evaluating oxidative stability during processing or storage. The thiobarbituric acid (TBA) assay is the most commonly-used spectrophotometric method determining MDA content[2]. By using TBA as a derivatizing agent, the MDA in the biological matrix can react with it and form an MDA-TBA adduct with a maximal absorption wavelength at 532 nm. However, the TBA assay has limitation on detection specificity, since thiobarbituric acid reactive substances (TBARS) such as other aldehydes, sugars, amino acids, and indigenous pigments in matrix can also react with the TBA, and exhibiting a similar absorption wavelength which could cause false-positive results. This assay limitation could get worse at the derivatization process for forming MDA-TBA adduct (95–100 °C, pH is about 4)[3]. Therefore, the TBA assay over-estimates the real total MDA compounds (tMDA) content. Chromatographic techniques were thus applied due to a better separation capacity avoiding false signals from the spectrophotometric detection. In present, there are over 10 kinds of detectors, dozens of detection methods with various hydrazine-based derivatizing agents (2,4-dinitrophenylhydrazine (DNPH), perfluorophenylhydrazine (PFPH), etc.) used in MDA quantification. Some researchers optimized the chromatographic assays for an improved volatility and stability in terms of specific samples. For instance, the modified high performance liquid chromatography-ultraviolet (HPLC-UV) detection assay was used for MDA detection in fish livers after derivatization with DNPH, showing an detection limit up to 0.39 μmol/L, with a high recovery in range of 92.4%–104.2%[4]. An improved ultra-high performance liquid chromatography-high resolution mass spectrometry (UPLC-HRMS) assay was used for identifying both tMDA and free MDA (fMDA) content in plasma under derivatization with DNPH, showing the lowest limit of quantification (0.1 μmol/L) and high accuracy (101%–107%) at low and high concentrations, respectively[5], HPLC-fluorescence (FL) detection for MDA-TBA adduct in various biological samples was also reported with a high recovery (91.2%–107.6%) and requiring a concentration of 0.15–3.0 μmol/L[6]. In comparison to HPLC, gas chromatography-mass spectrometry (GC-MS) assay is more frequently used for MDA quantification due to higher sensitivity for volatile compounds with a smaller molecular weight. For instance, Cheng et al.[7] applied the thermal desorption-GC-MS on tilapia for evaluating the oxidation induced flavor formation mechanisms under cold storage. Zhou et al.[8] applied GC-MS to monitor the

odor content fluctuation under the freeze-thaw cycles and heat treatment on frozen grass carp. Although researchers reported detection of MDA-PFPH adduct in raw and cooked pork meat with an fortified specificity and validation with GC-MS against strong acids and temperature in conventional MDA assays[9], limited studies were conducted on evaluating with the improvement under advanced GC-MS derivatization assays for fish products.

In food matrix like meat, as fMDA can form Schiff-base adducts with amino groups on suspectable membrane protein residues including lysine, arginine and proline, the bound MDA (bMDA) become the majority in meat products[10]. Recent paper revealed a binding mechanism on the interaction of MDA and rabbit meat myofibrillar protein[11]. Therefore, the hydrolysis treatment before derivatization and GC-MS detection is critical to assay accuracy. Hydrolysis treatment is capable to break down the covalent linkage between the meat proteins and MDA, release more fMDA with the carbonylation of proteins[12]. Current hydrolysis in the assay commonly applied 5–10 g/100 mL trichloroacetic acid (TCA) solution[13–14]. While it also can precipitate proteins in the hydrolysate. Supportively, a clinical study found that the MDA readings achieved after TCA hydrolysis were much lower than that hydrolyzed by NaOH[15]. Similarly, the MDA-fatty acid-free bovine serum albumin (BSA) adduct hydrolyzed by 0.5 mol/L H_2SO_4 for 60 min or 1 mol/L HCl for 120 min could reach equivalent MDA result in comparison with the hydrolysis treatment under 1 mol/L NaOH at 60 °C for 60 min[16]. The less hydrolysis efficiency under TCA hydrolysis might be related with short treatment time and low concentration comparing with alkaline hydrolysis. Another potential result-suppressing reason from acid hydrolysis in MDA determination might be related with the Hock rearrangement process with the acid-catalyzed reaction involving organic hydroperoxides, leading to less MDA formation during hydrolysis[17]. Alkaline hydrolysis accompanying with a heating effect might lead to a higher MDA content due to the thermal lipid oxidation. Therefore, some assay optimization studies applied antioxidant such as 2,6-di-tert-butyl-4-methylphenol (BHT) before hydrolysis[6,18]. However, other studies have shown that hydrolysis without BHT would not influence the value[19]. Therefore, it is important to confirm whether the lipid oxidation promoted by acid hydrolysis or alkaline hydrolysis would cause significant difference with the addition of BHT.

Above all, the present study compared two hydrolysis methods on Schiff-base adduct of bMDA, and an advanced assay was developed in aim to accurately measuring the MDA content in surimi foods as one example of typical aquatic foods. The paper also established three surimi sample models for comparing hydrolysis efficiency, investigated the effect of lipid oxidation during alkaline hydrolysis on the accuracy of results under assays. The subsequent extraction and derivatization procedures prior to the final GC-MS quantification were also further optimized for a better recovery of the MDA quantification.

2 Materials and methods

2.1 Materials and reagents

Fresh grass carp (*Ctenopharyngodon idella*) was purchased from local market in Hangzhou. After slaughtering in lab, the fish fillets were taken off and ground into surimi (sample A). The 1 000 g of sample A was then incubated with 1.5 L of 5 μg/mL MDA solution for 24 h, and the liquid was then removed by 4 °C centrifugation at 10 000 r/min for 15 min. The incubated surimi was divided into two parts: one was remained untreated (sample B), and another was defatted with subcritical extraction (sample C) according to the method described by Liu et al.[20], the collected two-phase (water and oil) liquid was then centrifuged at 10 000 r/min for 5 min (4 °C), the upper oil layer was then collected after a liquid-liquid separation. Five volumes of deionized water were mixed with the collected fish oils and separated from water phase for 5 times to completely removing MDA from the fish oil, anhydrous sodium sulfate was then applied for the MDA-free fish oil for water removal. The series of surimi samples (sample D) were then prepared by adding the MDA-free fish oil into the untreated surimi (sample A) with 4 different oil-to-surimi mass ratios (*m*/*m*), specifically 0% group (surimi without the addition of fish oil), 2.5%, 5%, and 10% groups (surimi added with 2.5%, 5%, and 10% fish oils).

All chemical reagents used in the study were of analytical or HPLC grade with highest purity. 1,1,3,3-Tetraethoxypropane (TEP), 1,1,3,3-tetraethoxypropane-D_2 (TEP-D_2), BHT, TCA, disodium hydrogen phosphate dodecahydrate, citric acid monohydrate, anhydrous sodium sulfate, *n*-hexane, MeOH, and acetonitrile (ACN) were purchased from Aladdin (Shanghai, China), PFPH were purchased from Sigma (St. Louis, USA). HCl (mass fraction 37%) and sodium citrate were purchased from Xilong Science (Shanghai, China). The concentrated MDA or MDA-D_2 solution was prepared by reacting with 60 μL of TEP or TEP-D_2 solution with 25 mL 0.1 mol/L HCl in at 40 °C for 3 h, then diluted to 20 μg/mL and stored at 4 °C. The pH 3.0 buffer solutions were prepared by 0.1 mol/L citric acid and 0.2 mol/L disodium hydrogen phosphate.

2.2 Hydrolysis treatments

For acid hydrolysis, 2 g of sample was added with 100 μL 2 mg/mL BHT and 8 mL TCA solution with different concentrations (2.5, 5, 7.5, 10, and 15 g/100 mL). After blending for 30 min, the hydrolyzed mixture was centrifuged at 10 000 r/min for 5 min. After filtration, and 4 mL of the filtrate was transferred into a centrifugal tube, added deionized water to reach a final volume of 5 mL, and 3 mL buffer solution (pH 3.0) was added to maintain a stable acidic condition. Distilled water instead of TCA solution was used as a blank.

For alkaline hydrolysis, 2 g of sample was added with 100 μL 2 mg/mL BHT and 8 mL NaOH solution with different concentrations (0.1, 0.25, 0.5, 1, and 2 mol/L), after reaction at 60 °C water bath for 60 min. The pH of the solution was adjusted to 3.0 with addition of 0.1 mol/L HCl, then deionized water was added to reach a final volume of 9 mL. The mixture was centrifuged at 10 000 r/min for 5 min, and after filtration, 4.5 mL of the filtrate was mixed with 3.5 mL buffer solution (pH 3.0). Distilled water instead of NaOH solution was used as a blank.

2.3 Derivatization with PFPH

Based on former works, derivatization of MDA promotes the sensitivity of MDA quantification[21]. To detect the MDA and MDA-D_2 derivatized adducts, 2 mL of the acid or alkaline hydrolysate was directly reacted with 100 μL 2.5 μmol/L PFPH solution at 50 °C for 60 min. After cooling, 2 mL of *n*-hexane was added for liquid-liquid extraction of MDA-PFPH and MDA-D_2-PFPH adducts. A total 1 mL of upper *n*-hexane layer was

then collected and dehydrated by anhydrous sodium sulfate. After 0.45 μm filtration, the derivatives were analyzed by a gas chromatography-mass spectrometer (Thermo Scientific Trace-1300, ANPEL Laboratory Technologies (Shanghai) Inc., Shanghai, China) equipped with electron ionization sources coupled with an Agilent DB-5 silica capillary column (60 m × 0.32 mm, 1.00 μm) (Shanghai Gaoge Industry and Trade Co., Ltd., Shanghai, China). Operation conducted under a helium (99.9%), flow rate 1.0 mL/min, the initial column temperature set at 280 °C, and increased by 15 °C/min. Then column temperature remained at 250 °C for 8 min. The entire operating time took about 23.3 min. The mass selective detector conditions involved a capillary direct interface temperature at 250 °C with 70 eV ionization energy. Full-scan mass spectra (*m*/*z* 40–500) were recorded for individual volatile compounds. The retention time for MDA-PFPH and MDA-D_2-PFPH were identified at 12.31 min. The derivatives were analyzed by GC-MS under the selected ion monitoring mode, and ions of *m*/*z* 234 and 236 were selected for quantifying the MDA-PFPH and MDA-D_2-PFPH compounds, respectively.

2.4 Optimization on liquid-liquid extraction and acid precipitation

To further improve the sensitivity of the alkaline hydrolysis-based MDA quantification method, four optimization strategies were further conducted in considering solvent effect during liquid-liquid extraction process and the following protein precipitation from different acidifying treatment prior to liquid-liquid extraction. As showed in Figure 1, methods A and B evaluated the effect from solvents in liquid-liquid extraction, 4 mL alkaline hydrolysate from sample A was firstly mixed with 8 mL ACN (method A) or MeOH (method B) to extract available MDA from the hydrolysate. After vortex and setting, the upper liquid layer was transferred and adjusted to pH 3.0, then the 2 mL pH-controlled MDA extracts were reacted with 100 μL 2.5 μmol/L PFPH for derivatization. To extract MDA-PFPH adduct, 2 mL *n*-hexane was then added into the ACN or MeOH extracts for separation. Methods C and D evaluated the effect from acid precipitation after the liquid-liquid centrifugation. Hydrolysate was regulated to pH 3.0 with 0.1 mmol/L HCl solution, and the deionized water was added to a final volume of 8 mL (method C). The pH of the alkaline hydrolysate was firstly adjusted to 4.0, then 20 g/100 mL TCA solution was added to adjust the pH to 3, and deionized water was added for 8 mL final volume (method D). After vortex and centrifugation, the 8 mL acid precipitated hydrolysate was collected and 2 mL of it was transferred into a centrifuge tube, mixed with 100 μL 2.5 μmol/L PFPH for derivatization. Then 2 mL of *n*-hexane was added for extracting available MDA-PFPH adduct.

To confirm the contribution of key factors on MDA-PFPH recovery rate of the methods, different pH (1.0, 2.0, 3.0, 4.0, 5.0, and 6.0), temperature (20, 30, 40, 50, 60, and 70 °C) and reaction time (30, 60, 90, 120, and 180 min) during the derivatization were compared individually. In addition, extra works were also done for method A regarding the effect of second extraction solvent (*n*-hexane, toluene, chloroform, ethyl acetate) and aqueous solution with different NaCl concentration (0, 1.25, 2.5, 5, 7.5, and 10 g/100 mL) were compared.

2.5 Determination of recovery rate and evaluation of assay sensitivity

To further confirm the accuracy and precision of methods A and D quantifying the real MDA content from untreated surimi (sample A), 100 or 500 μL of 10 μmol/L MDA solution was added to sample A respectively after hydrolysis to prepare standard addition samples. MDA quantification was determined three times a day and completed within two consecutive days for controlled sensitivity.

2.6 Statistical analysis

The results of MDA quantification peak area are expressed as mean ± standard deviation (SD), and analyzed using analysis of variance (ANOVA) to verify the significance differences of results among treatments using the *t*-test ($P < 0.05$).

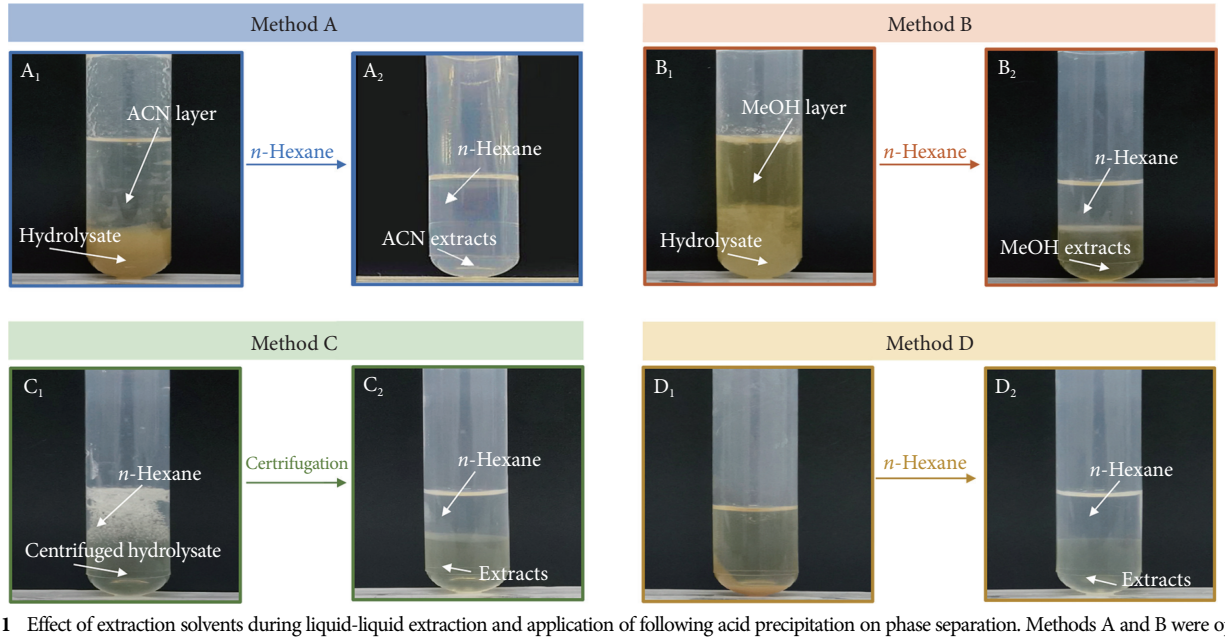

Figure 1 Effect of extraction solvents during liquid-liquid extraction and application of following acid precipitation on phase separation. Methods A and B were operated with the addition of ACN or MeOH and *n*-hexane during liquid-liquid extraction respectively for improving recovery of available MDA-PFPH adduct, methods C and D were conducted with acid precipitation by HCl only or HCl-TCA respectively for removing the signal interference from proteins.

3 Results and discussion

3.1 Hydrolysis efficiency between hydrolysis methods in three surimi models

As shown in Figure 2, Overall, alkaline hydrolysis was found leading to a higher MDA detection peak than TCA hydrolysis, the maximum peak area obtained by NaOH hydrolysis was about twice of that under the TCA hydrolysis for almost all three sample models.

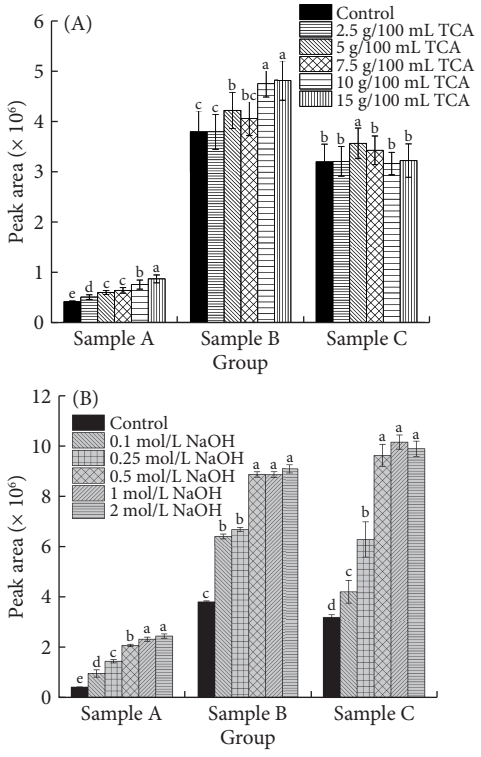

Figure 2 Effect of solvent concentration during (A) TCA hydrolysis and (B) NaOH hydrolysis on final MDA quantification for three surimi sample models (sample A: untreated surimi, sample B: MDA incubated surimi, sample C: fat-free MDA incubated surimi). The control was prepared with distilled water instead of TCA/NaOH hydrolysis. For each sample model, different lowercase letters above the histograms indicate significant differences among hydrolysis solvent concentrations ($P < 0.05$).

For untreated surimi after TCA hydrolysis, the peak areas of MDA signal in GC-MS were increased with the elevated TCA concentration (0 to 15 g/100 mL). While the peak areas gradually increased and stayed stable with the increase of NaOH concentration under the alkaline hydrolysis. For MDA incubated surimi prepared with a controlled high oxidation level, both hydrolysis methods caused a significant higher MDA content in comparison with other two sample models. Although the MDA content in MDA incubated surimi was lower than that in fat-free MDA incubated surimi at high NaOH concentration, the difference was not significant. However, the higher hydrolysis efficiency was found when TCA concentration reached 15 g/100 mL or NaOH concentration reached 0.5 mol/L. Considering the fact that the high MDA content under alkaline hydrolysis could also be due to the occurrence of thermal lipid oxidation during alkaline hydrolysis and longer reaction time[22]. Our hydrolysis treatments both added the BHT for avoiding the bias signals from lipid oxidation induced by processing. And our preliminary studies showed no significant difference on MDA content among untreated surimi with the addition of 0, 2, 20, and 200 mg/mL BHT (data not shown). To better clarify the hydrolysis efficiency improvement effect under alkaline hydrolysis without the presence of fish oils in surimi, the fat-free MDA incubated surimi was prepared by fat removal (1.5% oils remained) using subcritical extraction and controlling MDA contents. Our preliminary study confirmed that 1.5% fish oils remained in defatted surimi would not affect the MDA content comparing with the complete fat-free samples (data not shown). For fat-free MDA incubated surimi, our results showed that the peak areas were gradually stable with the increase of NaOH concentration under alkaline hydrolysis, which is similar to the trends for other two samples. This is another strong evidence showing that high hydrolysis efficiency on Schiff-base adduct of MDA in food matrix occurred with high concentration of NaOH. For TCA hydrolysis, the peak area of fat-free MDA incubated surimi exhibited a different changing pattern with increased concentration, only exhibited higher peak areas under 5 g/100 mL TCA hydrolysis. The reason might be that the high concentrated TCA solution can promote the reaction between MDA and other components, resulting in the overall reduction of the peak area. In addition, under alkaline hydrolysis, the peak area of fat-free MDA incubated surimi was found higher than that of MDA incubated surimi when over 0.5 mol/L NaOH was used as hydrolysis reagent. A reasonable explanation for this phenomenon could be that although fat-free MDA incubated surimi contained less MDA Schiff-bases than MDA incubated surimi in terms of the same mass, the excess MDA might be produced from lipid oxidation during high-heat alkaline hydrolysis in comparison to the fMDA released through TCA hydrolysis.

In addition, it was reasonable that the MDA peak areas of untreated surimi and MDA incubated surimi shared same changing trend with increasing concentrations of TCA and NaOH solutions, while it was obvious that different hydrolysis efficiency were presented for fat-free MDA incubated surimi (avoiding the induced lipid oxidation from high fat content in matrix). Specifically, the TCA concentrations did not influence hydrolysis efficiency, while NaOH concentrations exhibited similar influence like in untreated surimi and MDA incubated surimi. The conflict results indicated that the MDA detection under alkaline hydrolysis is more consistent in different surimi models, showing the highest release of fMDA at 1 mol/L NaOH.

3.2 Detection accuracy between hydrolysis for surimi with different fish oil levels

As shown in Figure 3, to evaluate the MDA detection accuracy with different concentrations for two hydrolysis methods regarding the oxidative levels of surimi samples, MDA peak areas in sample D, the untreated surimi with the addition of 0%–10% fish oils were compared. For TCA hydrolysis (Figure 3A), the peak areas of samples containing 5% and 10% fish oil were significantly higher than the control samples (0%). The increased MDA peak areas could be attributed to the fact that TCA could aggravate the Hock cleavage of hydroperoxides formed in surimi with high content of unsaturated fat[23]. This exhibited the fact that the presence of oils in surimi samples facilitated the bias readings of MDA no matter the

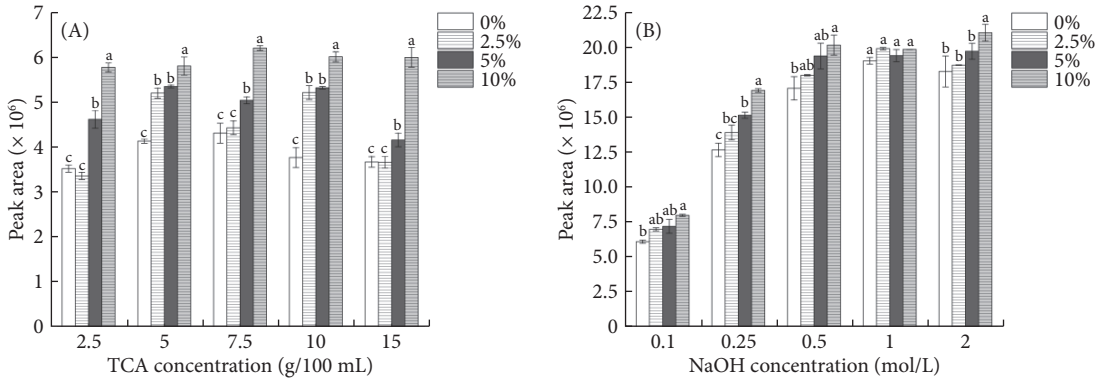

Figure 3 Effect of added fish oil content (sample D, surimi added with 0%, 2.5%, 5%, and 10% MDA-free fish oils) during (A) TCA hydrolysis and (B) NaOH hydrolysis under increasing concentration on final MDA quantification. Different lowercase letters above histograms under each concentration of the hydrolysis method indicate significant differences among surimi samples with different fish oil content ($P < 0.05$).

concentration of TCA reagents. And it was also noticed the levels of lipid oxidation detected presenting proportional to the content of added fish oil under TCA concentration, especially at 15 g/100 mL with the highest hydrolysis efficiency according to former session. For alkaline hydrolysis (Figure 3B), under high concentrated NaOH (1–2 mol/L) hydrolysis which holding the highest hydrolysis efficiency, the levels of lipid oxidation in samples were almost not affected by added oils. In summary, TCA solution would easily catalyze the reaction of MDA with reactive substances from oil components, thus exhibiting more severe lipid oxidation results comparing with the alkaline hydrolysis. Therefore, detection accuracy under alkaline hydrolysis is better than acid hydrolysis. Since the detection of MDA in samples with different oxidative level is lack of sensitivity, the following works further optimize the extraction and derivatization process under the alkaline hydrolysis.

3.3 Optimization results on liquid-liquid extraction and acid precipitation protocols

Optimization on MDA assay with PFPH derivatization targeting sensitivity improvement included the evaluation under effect of first extraction, derivatization and second extraction protocols.

Different with tradition TCA hydrolysis, whose hydrolysate pH could shift into around pI of the fish muscle proteins (pH 3.6–6) in the aqueous phase[24], the hydrolysate after alkaline hydrolysis using 1 mol/L NaOH for 60 min might promote more proteins dissolved in the hydrolysate. It is unfavorable for the subsequent detection since the soluble proteins would interfere the real signal during MDA quantification. Therefore, the hydrolysates must be extracted by organic solvent before detection (first extraction process). Since protein could precipitate in ACN solution, some studies reported extracting MDA from alkaline hydrolysate with ACN[10]. However, the poor polarity of ACN also might lead to low extraction recovery of MDA from buffer hydrolysate, thus inducing lower detection sensitivity. Therefore, more polar solvent such as MeOH was also used in our comparison experiment even it might have a slightly weaker protein precipitation capacity. Figures $1A_1$ and B_1 exhibit the exact protein precipitation status after adding ACN and MeOH, respectively. After precipitating the protein, the MDA-PFPH derivatives then must be extracted by less polar *n*-hexane during the second extraction process. And it was obvious that under method A, no visible emulsion formed after both first and second extraction, the two-phase boundary in final extraction was very clear after vortex and standing (Figure $1A_2$), indicating the ACN then *n*-hexane extraction effectively avoided the interference of protein. In the method B, the yellow MeOH layer indicated that part of the soluble proteins in alkaline hydrolysate was transferred into the first extraction reagent (Figure $1B_1$) and facilitating the formation of surfactant layer between the MeOH and *n*-hexane phases after the second extraction (Figure $1B_2$). In conclusion, the use of ACN prior to *n*-hexane improved the sensitivity of assay.

Besides, mixing the alkaline hydrolysate with the acid buffer first to pH 3 before extraction process might be a promising alternative. Figures 1C and D show that some proteins precipitated with the addition of HCl. There were still some soluble proteins remained in solution after centrifugation and got suspended in the aqueous phase after the addition of *n*-hexane, the phenomenon last long time of standing (Figure $1C_1$). Meanwhile, there was an unclear surfactant layer between extracts and *n*-hexane phases, indicating poor separation capacity of method C (Figure $1C_2$). Instead, method D relying on the fact that TCA also had strong protein precipitation capacity and acidity like HCl, it was assumed that the use of HCl-TCA might achieve improved protein precipitation. Figure $1D_1$ shows that the two-phase boundary is clear after mixing and standing in comparison to method C.

3.4 Optimization on derivatization protocols

Since both method A and method D reached acceptable protein precipitation, their method sensitivity was further evaluated for the process of derivatization (Figure 4). Considering the factors influencing the assay sensitivity might be related with the low solubility of MDA in ACN, the reaction affinity between MDA and PFPH in ACN solution, and the presence of the MDA-PFPH derivatives in final *n*-hexane. Two methods were further optimized individually for the derivatization condition. For method A, the tested derivatization factors included pH, temperature, and time. Our results showed that the maximal peak area of MDA derivatives was generated at pH 2.0, 50 °C, and 60 min (Figure 4A). Since the only solvent during second extraction for extracting MDA derivatives from ACN was *n*-hexane, there was no comparison on the use of extraction solvents. For method D, besides the yield of MDA derivatives, the extraction efficiency might be affected by the ionic strength in the original alkaline hydrolysate and the type of second-extraction solvent. As shown in Figure 4B, the recovery rate of MDA derivatives reached the highest when derivatization at

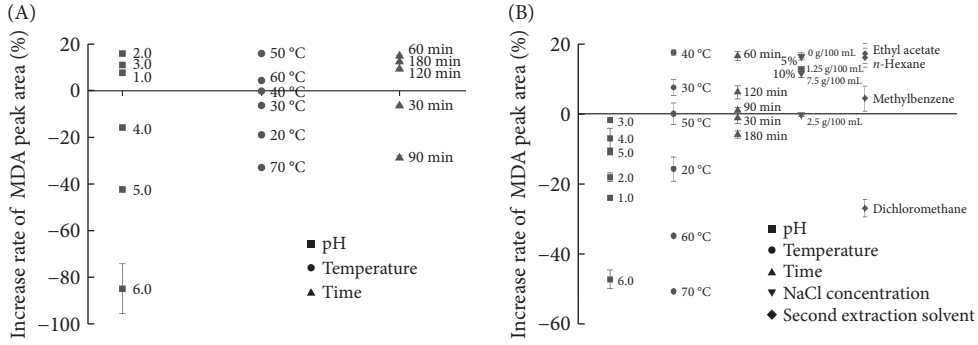

Figure 4 Effect of derivatization conditions after alkaline hydrolysis on increased rate of MDA peak area. (A) Method A (addition of ACN and *n*-hexane during liquid-liquid extraction) controlling different pH, temperature, and time; (B) Method D (HCl-TCA precipitation and solvent extraction prior to final detection) controlling different pH, temperature, and time, applied NaCl concentration and second extraction solvent. The increased rate of MDA peak area was based on the ratio of reading from the targeted condition to the original derivatization condition; the original method A derivatization is pH 3.0, 50 °C, and 60 min; the original method D derivatization is pH 3.0, 50 °C, 30 min, 0 g/100 mL NaCl, and *n*-hexane.

pH 3.0, 40 °C, and 60 min, without ionic strength (0 g/100 mL NaCl). And considering the presence of MDA-PFPH in ethyl acetate and *n*-hexane both could reached the highest recovery rate and with no significance, *n*-hexane was selected for extracting MDA-PFPH during second extraction.

Our results also indicated that the increased recovery rate of MDA under method D was about 41 times of that under method A, therefore, method D was selected as the optimal treatment prior to MDA analysis. To explore the mechanism behind the poor performance of method A. Researchers performed 4 sets of comparative experiments regarding the method sensitivity, including the MDA extraction efficiency of ACN, reaction activity of MDA-PFPH derivatization in ACN, and extraction efficiency of MDA derivatives from ACN with *n*-hexane. It was also found that the even 10% ACN extraction was applied as first extraction solvent, there was negligible effect on the derivative reaction activity and extraction efficiency with *n*-hexane extraction. Besides, the MDA quantification with ACN solution applied in first extraction for alkaline hydrolysate with a 1:2 volume ratio was found only reached 30% the recovery for those diluted with 1:3 volume ratio. In addition, the mass ratio of MDA-D_2 to PFPH in aqueous solution was around 24 times of which detected in ACN extract. And the peak area of MDA-D_2-PFPH extracted from aqueous solution was about 6.7 times that of extracted from ACN. Conclusively, all three steps under ACN could reduce the sensitivity of the method, especially when conducting the derivatization in ACN solution. Therefore, the method D was suggested with high assay sensitivity.

3.5 Precision and recovery evaluation

Figure 5 shows that HCl-TCA acid precipitation method has more advantages on recovery rate over ACN extraction method. The assay recoveries were 78.4%–94.7% under method A while 91.2%–102.1% under method D. The intra-day assay recovery and precision were 10.59% and 22.12% for method A, while only 3.12% and 4.29% for method D, respectively. Poor recovery and precision under ACN extraction method might be due to differences in the pH of samples, uneven dispersion of water components in the ACN extract, making it difficult to ensure the pH of the ACN extract remaining stable after pH adjustment, affecting the yield of the MDA derivatives.

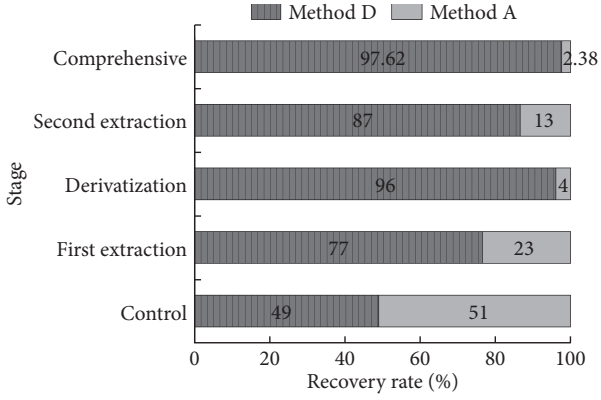

Figure 5 Recovery rate comparison between method A (ACN-*n*-hexane extraction) and method D (HCl-TCA acid precipitation) during different stage. Recovery rate was calculated as a sum ratio of MDA peak areas under method A and method D, and modifying to 100% in total.

Overall, it was concluded that method A showed a stronger protein precipitation ability but worse assay sensitivity, precision, and recovery in comparison with method D. Based on comprehensive considerations, HCl-TCA acid precipitation method was selected as the most optimal assay protocol for detecting MDA-PFPH from alkaline hydrolysate from this study.

4 Discussion

Regards to the hydrolysis efficiency comparison test between two hydrolysis methods, our finding showed significant increase on MDA detection after samples treated by alkaline hydrolysis over traditional TCA hydrolysis, especially for those surimi samples already in later oxidation stage. Our former review also comprehensively summarized analytical methods for detecting MDA, 4-hydroxy-nonenal dominant oxidative indicators in food matrix, and pointed out that the reasons for higher hydrolysis efficiency under NaOH could be due to its effective breakage capacity of bMDA[22]. Although some studies evaluated the application of alkaline hydrolysis on MDA quantification improvement in terms of fortified release of fMDA from human plasma samples using HPLC or GC-MS[25–26], limited studies is available evaluating its improvement on increased efficiency considering fresh, oxidative and oxidative defatted samples yet. This study considered the reason of variation on hydrolysis efficiency with two different hydrolysis reagents as liability to the break of the

MDA complex bondage in sample matrix. Considering surimi sample itself could turn into a more melted appearance after NaOH hydrolysis at higher temperature, the MDA Schiff-base was able to react with NaOH more easily in aqueous base. However, the hydrolysis efficiency between TCA and MDA Schiff-base relied on the sample sizes and surface areas facilitating the interactions between solvent and surimi samples. From our accuracy comparison test between acid and alkaline hydrolysis, it was noticed the high content oils in fish samples contributed more bias readings, especially under traditional TCA hydrolysis method. To extend the assay on determining MDA in meat products in addition to the fish meat, the oil content and high unsaturated fat composition difference must be considered as they might be less oxidative with the lower polyunsaturated fat[27]. The later sessions investigated the effect of first extraction solvent and the presence of different acid precipitation treatment, it was found the ACN has excellent protein precipitation capacity while worse extraction efficiency on fMDA, this could be due to the difficulty of protonation of MDA in nonpolar solvent. Based on our results, it is believed that the change of derivatization reagent or conditions will not change the result, since the content of MDA derivatives in ACN is also significantly lower than that in water. Considering more than 90% of the bMDA in biological matrix are in form of proteins and less than 10% are in the form of lipids[5], the poor solubility of the released MDA in ACN makes it reasonable to the low sensitivity. Some studies also reported that fMDA could not be preserved stably in the ACN extracts containing NaOH solution[28]. In terms of acid precipitation as alternative for protein precipitation of alkaline hydrolysate among all assays we targeted in the paper, it exhibited the best sensitivity, recovery, and precision. In fact, the application of 20 g/100 mL TCA for acid precipitation on fMDA and bMDA from alkaline hydrolysate of plasma samples has started to operate in UPLC-HRMS assay[5]. In the future, the other high resolution chromatographic assays targeting determination of both bMDA and fMDA quantification at the same time for meat products could be further explored based on presented optimal protocol. Besides, more efforted could be focused on the use of novel solvent free detection system or protocols with less sample sizes for an extended application for evaluating the MDA or oxidation levels in fish products[29].

5 Conclusion

This study comprehensively developed an advanced GC-MS method quantifying the MDA content in fish products, expected to best release the bMDA in surimi matrix without significant aggravation of lipid oxidation under alkaline hydrolysis treatment. The surimi hydrolyzed by 1 mol/L NaOH at 60 °C for 60 min was proved reached its highest hydrolysis efficiency, its MDA recovery reached twice of that under 15 g/100 mL TCA hydrolysis. It was also confirmed that the amount of excess MDA produced by processing lipid oxidation during alkaline hydrolysis was less than that under high concentrated TCA. Overall conclusion suggested alkaline hydrolysis promoting the MDA recovery no matter the fat content in samples, and effectively avoiding bias signals from the processing oxidation and reaction of MDA and other reactive substance. To most improve the sensitivity and precision of detection assays, we investigated optimal extraction treatments and acid precipitation prior to extraction to reduce pollution of fish proteins remaining in the alkaline hydrolysates, it was determined that method D applying HCl and TCA adjusting solution pH 2–3 was able to precipitate proteins as well as preserve accuracy. Since fMDA is less stable in alkaline environment, it is very necessary to convert MDA into stable MDA derivatives after alkaline hydrolysis prior to GC-MS analysis for better recovery and sensitivity. In the future, the application of this assay could be extended to other meat products with different oxidative liability due to different fatty acid distribution.

Conflicts of interest

The authors declare that they have no conflict of interest.

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