

Analysis of the effects of different protease types on volatile compounds and oxidative properties of blackthroat seaperch (*Doederleinia berycoides*) liver oil

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Abstract: This study evaluated the effects of different proteases on the extraction, flavor profile, and oxidative stability of blackthroat seaperch (*Doederleinia berycoides*) liver oil obtained by aqueous enzymatic extraction. Trypsin-assisted extraction yielded oil with the greatest oxidative stability. Combined analysis by gas chromatography-ion mobility spectrometry and gas chromatography-mass spectrometry identified aldehydes as the characteristic volatile compounds in the liver oil. The relative odor activity value analysis identified (*E*)-2-nonenal, furaneol, benzeneacetaldehyde and 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-buten-2-one as the key volatile compounds. Differences in oxidative stability and thermal behavior were observed among the extraction methods, with the antioxidant capacity of blackthroat seaperch liver oil following the order of pepsin (PS) > alkaline protease > trypsin (TS) > neutral protease extraction methods. Differential scanning calorimetry analysis revealed that PS melting and cooling profiles enhanced flavor release and persistence, while TS and PS exhibited ω -3 fatty acid pyrolysis. These findings demonstrate the importance of enzyme selection for liver oil flavor and stability and support the valorization of blackthroat seaperch by-products.

Keywords: blackthroat seaperch liver oil; enzymatic extraction; volatile compound; antioxidant activity; thermal behavior

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1 Introduction

In recent years, the increasing public interest in health and nutrition has intensified the demand for high-value functional foods. Aquatic foods, caught or farmed in freshwater and marine ecosystems, play a central role in food systems globally and contribute to nutrition security for billions of people^[1]. As a high-fat white meat fish, the blackthroat seaperch (*Doederleinia berycoides*) is mainly distributed from the Eastern Indian Ocean to the Western Pacific Ocean and is commonly found in the waters around the East China Sea, Japan, and Australia. As an important economically valuable fish species in the East China Sea, it has gained wider recognition in the market in recent years with the popularization of ultra-low temperature freezing technology. The muscle and liver of blackthroat seaperch are rich in lipids, which may increase their susceptibility to lipid oxidation and spoilage, affecting its storage stability and processing application. Currently, liver by-products are often discarded during processing, leading to substantial resource loss^[2]. Converting these

by-products into fish oil presents a promising strategy for valorization.

Fish oil is rich in fatty acids, which are more readily absorbed by the human body than plant-derived components and help enhance memory^[3]. Wu et al.^[4] reported that fish oil extracted from the liver of the blackthroat seaperch was rich in essential fatty acids and unsaturated fatty acids, and evaluated its lipid oxidation level and fatty acid composition. The results indicated that blackthroat seaperch liver oil is a high-quality fish oil, and it provides a unique contribution to the lipid volatile flavor. The fatty acid composition of fish oil is particularly rich, containing mainly monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs), which are essential for human health^[5]. However, to fully transform the nutritional potential of blackthroat seaperch liver oil into practical product value, further research on the extraction methods of the fish oil remains necessary. Across the fish oil industry chain, it is necessary to further industrialize and strengthen research and development capabilities in order to meet the demand for

large-scale production and maximize the value of fish oil resources^[6]. Currently, the common methods for extracting fish oil from by-products in the industry mainly include pressing and organic solvent extraction^[7]. These two methods typically use organic solvents such as ether, acetone, acetonitrile, and methanol to extract the oil and may leave residual organic reagents during the extraction process, leading to contamination of fish oil products and negatively affecting food safety^[8]. The traditional solvent extraction process carries the risk of chemical residues, and it is difficult to precisely control the flavor of the oil. Furthermore, prolonged high-temperature heating accelerates lipid oxidation, particularly in PUFA, and causes protein denaturation, which may compromise the nutritional and functional properties of fish oil as well as the bioavailability of proteins. Therefore, there is an urgent need to develop green and efficient enzymatic extraction technologies to mitigate oxidative off-flavors, improve product quality, and promote the development of the aquatic processing industry toward high-value-added products^[9].

Hydro-enzymatic extraction, as a green and efficient technique, utilizes the separation of oil by leveraging differences in affinity between oil and non-oil components as well as the density difference between oil and water^[10]. Employing cellulase, protease, and other composite enzyme systems to specifically degrade cell wall structures and lipoprotein complexes significantly enhances the extraction efficiency of blackthroat seaperch liver oil and improves the retention of active ingredients through specific enzyme-substrate interactions^[11]. The interaction between the enzymatic environment and the substrate specificity of proteases may alter the oxidative stability of blackthroat seaperch liver oil by modifying lipid release patterns and the retention of endogenous antioxidants. Differences in the fatty acid composition of enzymatically extracted products not only determine their oxidative susceptibility but may also influence lipid crystal network formation through intermolecular forces, thereby modulating the encapsulation and release kinetics of volatile flavor compounds^[12].

This study focused on the product characteristics of blackthroat seaperch liver oil obtained through different extraction processes, aiming to investigate the effects of various proteases on the extraction yield, bioactive components, fatty acid composition, and volatile flavor of the liver oil. Gas chromatography-ion mobility spectrometry (GC-IMS) and gas chromatography-mass spectrometry (GC-MS) combined with relative odor activity value (ROAV) techniques were used to screen key flavor compounds in fish oils from different sources and to explore the regulatory mechanisms of enzymatic processes on blackthroat seaperch liver oil volatile compound formation. The oxidative stability was evaluated using the Rancimat method, antioxidant activity was assessed via 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) cation radical scavenging assays, and thermal characteristics kinetics were analyzed by differential scanning calorimetry (DSC). These multidimensional analyses revealed the specific effects of protease types on the oxidative degradation pathways, radical scavenging efficacy, and thermodynamic behavior of the blackthroat seaperch liver oil, providing a data-driven basis for the optimization of the enzymatic process. Furthermore, the influences of different enzyme types on extraction efficiency, fatty acid composition, and volatile flavor profile were studied to achieve efficient extraction of blackthroat seaperch liver oil while improving its volatile flavor quality.

2 Materials and methods

2.1 Liver oil extraction

Blackthroat seaperch samples were purchased from Zhoushan International Aquatic Trade Zone (Zhoushan International Aquatic City, Zhoushan, China), and transported to the laboratory under frozen conditions at -18°C within 0.5 h. The samples were thawed at 4°C , after which the fish livers were homogenized for 60 s. Distilled water was added at a solid-to-liquid ratio of 1:5 (g/mL), followed by pulping for 30 s. The pH was adjusted to the optimal value for each protease using HCl or NaOH. The liver oils extracted using different proteases were designated as follows: pepsin (PS) ($\text{pH } 2.1 \pm 0.2$), trypsin (TS) ($\text{pH } 8.2 \pm 0.1$), neutral protease (NP) ($\text{pH } 7.0 \pm 0.1$), and alkaline protease (AP) ($\text{pH } 10.0 \pm 0.4$)^[13]. The slurry was transferred into 50 mL centrifuge tubes, and proteases were added at 1% (*m/m*) relative to the substrate. Enzymatic digestion was performed at 37°C for 40 min in a constant-temperature shaker operating at 100 r/min. After digestion, the tubes were centrifuged at 8 000 r/min for 20 min, and the upper clear phase was collected as crude fish liver oil. Liver oil extraction rate was calculated with formula (1):

$$\text{Extraction rate (\%)} = \frac{m_0}{m} \times 100 \quad (1)$$

Where m_0 is the crude fish liver oil mass (g); m is the sample mass (g).

All reagents used in this experiment were analytically pure and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2 Determination of squalene, vitamin A, and sterol contents

The squalene content was determined according to the method described by Ying et al.^[14] with slight modifications. Briefly, 0.5 g of blackthroat seaperch liver oil was saponified with a potassium hydroxide-ethanol solution (1 mol/L). After extraction with *n*-hexane, the content was quantified by GC.

The vitamin A was determined by dissolving 50 g of KOH in 50 mL of ultrapure water, followed by cooling and storage. Vitamin C-ethanol solution was prepared by dissolving 2 g of vitamin C in 10 mL of ultrapure water and adding 90 mL of anhydrous ethanol. A 0.25 g portion of blackthroat seaperch liver oil was weighed, 100 mL of vitamin C-ethanol solution and 25 mL of KOH solution (50 g/100 mL KOH and 1 g/100 mL ethanolic pyrogallol) were added, and the mixture was saponified for 30 min. The saponification solution was extracted twice with 50 mL of petroleum ether, and the combined ether layers were washed with 100 mL of water until neutral. The ether layer was then passed through anhydrous sodium sulfate, concentrated to near dryness by rotary evaporation, and further dried under a gentle stream of nitrogen. The residue was reconstituted to a final volume of 10 mL with methanol, filtered through a 0.22 μm membrane filter, and subjected to instrumental analysis. For vitamin A analyses, a 10 μL sample was injected and separated by a C_{18} chromatographic column (4.6 mm $\times$ 150 mm, 5 μm , Agilent, USA) at 25°C . High performance liquid chromatography (U3000, Thermo Fisher Scientific, USA) mobile phase was methanol and water (96:4, V/V) at a flow rate of 0.8 mL/min.

The sterol content was determined using a colorimetric method with slight modifications. β -Sitosterol standard was dried to

constant weight, accurately weighed, and dissolved in hexane to prepare a 0.1 mg/mL solution. A 0.25 g portion of blackthroat seaperch liver oil was weighed, 25 mL of trichloromethane was added, and the mixture was subjected to ultrasonic treatment for 30 min, cooled to room temperature, shaken well and filtered. A 10 mL aliquot of the filtrate was accurately measured and concentrated to dryness under reduced pressure. The residue was refluxed with 25 mL of 10 g/100 mL sodium hydroxide-ethanol solution for 2 h, removed from the heat, and cooled to room temperature. Water was added, and the solution was transferred to a separatory funnel and extracted with *n*-hexane three times (20 mL each time). The *n*-hexane layer was combined and washed with water twice, and the aqueous layers were discarded. The *n*-hexane layer was concentrated and made up to 50 mL, and the resulting solution was shaken well to obtain the test solution. An appropriate amount of the standard or test solution was taken, 0.5 mL of 5% vanillin-glacial acetic acid solution and 0.7 mL of perchloric acid were added, and the mixture was heated in a 70 °C water bath for 30 min, then removed from the water bath, and cooled to room temperature. The volume was made up with glacial acetic acid, and the solution was shaken well for color development. The absorbance was measured at 543 nm. A corresponding reagent blank control was prepared, and the absorbance was measured at 543 nm. The sterol content was calculated using the formula (2):

$$W = \frac{(\rho - \rho_0) \times V \times N}{m} \quad (2)$$

Where *W* is sterol content (mg/kg); ρ is sterol concentration in the sample solution (mg/L); ρ_0 is sterol concentration in the blank control (mg/L); *V* is finished volume (mL); *N* is dilution factor; *m* is sample weight (g).

2.3 Determination of fatty acid content

Fatty acid methyl esterification was performed as previously described^[15]. Blackthroat seaperch liver oil (50 mg) was dissolved in ethanol and diluted to 25 mL. A 1 mL aliquot of this solution was transferred to a 15 mL centrifuge tube. The lipid extract was mixed with 8 mL of 2 g/100 mL sodium hydroxide-methanol solution, connected to a reflux condenser, and saponified in an (80 ± 1) °C water bath until oil droplets disappeared. Subsequently, 7 mL of 15 g/100 mL boron trifluoride-methanol solution was added through the top of the condenser, and reflux was continued for 2 min at (80 ± 1) °C. The condenser was rinsed with a small amount of water. After cooling to room temperature, 10 mL of *n*-heptane was added, followed by shaking for 2 min, and the mixture was then saturated with sodium chloride solution. After phase separation, the organic layer was dried over 3 g of anhydrous sodium sulfate. The supernatant was then transferred to an injection vial for GC-MS analysis.

2.4 Determination of volatile compounds by GC-IMS

Volatile compounds were analyzed using a modified method based on Chen et al.^[16]. A 2.0 mL aliquot of blackthroat seaperch liver oil was placed into a 20 mL headspace vial and analyzed with a FlavourSpec® flavor analyzer (G.A.S., Dortmund, Germany). Separation was performed on an MXT-WAX capillary column (30 m × 0.53 mm, 1.0 µm) with a total analysis time of 30 min. The lipid samples were incubated at 60 °C and agitated at 500 r/min for 15 min. Subsequently, 500 µL of the headspace gas was injected into the GC inlet using a heated syringe maintained at 85 °C, with an injection volume of 0.20 µL. High-purity nitrogen (99.999%) was

used as both the carrier and drift gas. The column was coupled to an IMS maintained at 45 °C. Data acquisition and compound identification were performed with dedicated software according to retention times and peak area ratios.

2.5 Determination of volatile compounds by GC-MS and ROAV analysis

Volatile compounds were analyzed by GC-MS according to the method described by Liu et al.^[17], with slight modifications. Briefly, 2.0 g of blackthroat seaperch liver oil was weighed and placed into a 20 mL headspace vial, followed by the addition of 4.0 mL of saturated sodium chloride solution. After sealing, the vial was equilibrated in a water bath at 80 °C for 20 min. A solid-phase microextraction fiber was then inserted into the headspace vial and maintained at 80 °C for 30 min to extract volatile compounds. The injection port temperature was set at 250 °C, and the desorption time was 5 min. High-purity nitrogen (99.999%) was used as the carrier gas at a flow rate of 1.0 mL/min. The GC oven temperature program was set as follows: the temperature was held at 50 °C for 2 min, increase to 180 °C at 5 °C/min and held for 5 min, and then increased to 250 °C at 10 °C/min and held for 5 min. The temperatures of the transfer line, quadrupole, and ion source were maintained at 280, 150, and 230 °C, respectively. Electron impact ionization was employed in full scan mode (*m/z* 40–600). Compounds were identified by comparing their mass spectra with those in the National Institute of Standards and Technology/Wiley libraries and were further validated using the linear retention indices of *n*-alkanes (C₈–C₂₀). The relative content was determined by peak area normalization.

ROAV was used to quantify the contribution of each compound to the overall flavor profile and was calculated using formulas (3) and (4):

$$OAV_i = \frac{C_i}{T_i} \quad (3)$$

$$ROAV = \frac{OAV_i}{OAV_{\max}} \times 100 \quad (4)$$

Where *OAV_i* is the odor activity value (OAV) of the compound *i*, *C_i* is the content of the compound *i* in the sample (µg/kg), and *T_i* is the odor threshold of the compound *i* (µg/kg). *OAV_{max}* is the maximum OAV of all compounds in each sample^[18]. Threshold data were obtained from publicly available databases and literature sources, including FlavorDB, PubChem, The Good Scents Co., and relevant scientific literature.

2.6 Determination of oxidative stability of liver oil by Rancimat method

The oxidation stability of the fish oil was determined according to the method described by Jiang et al.^[19] with appropriate modifications. Briefly, (3.0 ± 0.1) g of the fish oil sample was weighed into a reaction cell. The temperature was set at 90 °C, the air flow rate at 10 L/h, and the electrode cell was filled with 60 mL of ultrapure water. The results were expressed as the induction time in hours.

2.7 Determination of DPPH radical and ABTS cation radical scavenging rates

The DPPH radical scavenging assay was performed according to the method described by Bora et al.^[20] with appropriate

modifications. Briefly, the blank group (20 μ L of ethanol + 180 μ L of 0.1 mmol/L DPPH-ethanol solution), sample group (20 μ L of blackthroat seaperch liver oil + 180 μ L of 0.1 mmol/L DPPH-ethanol solution), and the control group (20 μ L of blackthroat seaperch liver oil + 180 μ L of ethanol solution) were added to a 96-well microplate. The reaction was carried out in the dark at room temperature for 30 min, after which the absorbance was measured at 517 nm using a microplate reader.

The ABTS cation radical scavenging assay was performed according to the method described by Ahmad et al.^[21] with appropriate modifications. Briefly, 7 mmol/L ABTS solution and 2.45 mmol/L potassium persulfate solution were prepared and mixed at 1:1 (V/V). The mixture was incubated at 25 °C in the dark for 12–16 h to generate the ABTS cation radical solution. The resulting solution was diluted with ethanol until an absorbance of 0.70 ± 0.02 was obtained at 734 nm and was stored in the dark until use. Fish oil dilutions were prepared as the test solutions. Subsequently, 20 μ L ethanol + 180 μ L ABTS cation radical working solution were added to the blank wells, whereas 20 μ L test solution + 180 μ L ABTS cation radical working solution were added to the sample wells. The mixtures were incubated at room temperature in the dark for 10 min, after which the absorbance was measured at 734 nm using a microplate reader.

2.8 Determination of thermal stability by DSC

The thermal characteristics of the samples were determined using a DSC Q1000 differential scanning calorimeter (TA Corp., Newcastle, USA) according to the method described by Ying et al.^[22] with appropriate modifications. A 25 mg sample of blackthroat seaperch liver oil was placed in a 100 μ L closed aluminum crucible, and the empty aluminum crucible was used as a blank control. The setup procedure was as follows: the initial temperature was 30 °C, the sample was heated to 600 °C at a rate of 20 °C/min and held for 5 min, then cooled to 30 °C at the same rate of 20 °C/min, and nitrogen was used as the carrier gas at a flow rate of 20 mL/min.

2.9 Data processing

Analyses were performed using OriginPro 9.1 (OriginLab, Inc., Northampton, USA). In order to statistically analyse the results of the experiments, the mean and standard error were calculated based on the results of three independent experiments. Differences in means were assessed using Duncan's multiple range test, and differences were considered significant at $P < 0.05$.

3 Results and discussion

3.1 Extraction rate of liver oil with different proteases

TS, used for the aqueous enzymatic extraction of blackthroat seaperch liver oil, yielded the highest extraction rate at 52.20%, followed by PS at 50.60%, NP at 47.90%, and AP at the lowest yield

of 41.10% (Table 1). Compared with our previous petroleum ether-based extraction method for fish liver oil, the aqueous enzymatic extraction approach exhibited significantly higher extraction efficiency (Table S1). The aqueous enzymatic method was chosen as a greener alternative to petroleum ether, owing to its greater safety^[23]. Additionally, the enzymatic digestion conditions (37 °C for 40 min) were milder in terms of temperature and duration compared to the chemical method, thereby reducing the risk of oil oxidation during extraction^[24].

3.2 Contents of squalene, vitamin A and sterol

Analysis of the data (Table 1) on vitamin A, squalene, and total sterols in blackthroat seaperch liver oil extracted by different proteases revealed that the vitamin A content in AP hydrolysis products reached 795.58 mg/kg, significantly higher than that in TS (516.64 mg/kg), NP (489.42 mg/kg), and PS hydrolysis products (387.07 mg/kg). This result may be closely related to the alkaline conditions under which AP functions. Vitamin A primarily resides within lipid structures, and its release requires disruption of cell membrane or lipoprotein stability^[25]. AP typically functions more effectively under higher pH conditions, which are more conducive to lipid emulsification or hydrolysis, thereby facilitating vitamin A dissolution. Furthermore, AP may exhibit a preference for cleavage sites on the carrier proteins binding vitamin A. Research by Fu et al.^[26] identified retinol-binding proteins, suggesting this mechanism could release greater amounts of free vitamin A. These findings are consistent with our results. The acidic environment of PS may limit the solubility of lipids, resulting in their least efficient extraction. In practice, with vitamin A as the main target component, the preference of AP can enhance the release of bioactive components from blackthroat seaperch liver oil by optimizing the reaction conditions.

Squalene, a natural antioxidant and immunomodulator, exhibited a different extraction efficiency trend across proteases. PS demonstrated the highest performance for this component, with an extraction yield of 689.37 mg/kg, slightly exceeding that of TS (684.42 mg/kg). In contrast, AP (643.46 mg/kg) and NP (645.23 mg/kg) showed relatively lower efficiency. This phenomenon is associated with squalene's molecular structure. Squalene is typically located within cell membranes or lipid droplets, and its release requires enzymatic cleavage of specific lipid bonds^[27]. PS functioning under acidic conditions, can more effectively degrade the protein matrix encapsulating squalene, thus releasing greater amounts of free squalene molecules. The reaction conditions for NP were insufficient to fully disrupt the binding between squalene and surrounding proteins or lipids, consequently resulting in lower extraction efficiency compared to the other groups.

The sterol data revealed relatively minor differences among the four proteases. NP (1.47%) and AP (1.46%) exhibited slightly higher extraction levels, while TS (1.44%) and PS (1.39%) yielded lower levels than those obtained with NP and AP. Sterols are a class of

Table 1 Extraction rate and bioactive components contents of blackthroat seaperch liver oil extracted by different proteases.

Items	PS	TS	NP	AP
Extraction rate (%)	$50.60 \pm 1.70^{\text{ab}}$	$52.20 \pm 1.30^{\text{a}}$	$47.90 \pm 1.50^{\text{b}}$	$41.10 \pm 1.20^{\text{c}}$
Vitamin A content (mg/kg)	$387.07 \pm 4.12^{\text{c}}$	$516.64 \pm 7.64^{\text{b}}$	$489.42 \pm 4.79^{\text{b}}$	$795.58 \pm 7.81^{\text{a}}$
Squalene content (mg/kg)	$689.37 \pm 7.05^{\text{a}}$	$684.42 \pm 6.97^{\text{a}}$	$645.23 \pm 7.56^{\text{c}}$	$643.46 \pm 6.99^{\text{a}}$
Sterol content (% <i>m/m</i>)	$1.39 \pm 0.60^{\text{a}}$	$1.44 \pm 0.60^{\text{a}}$	$1.47 \pm 0.50^{\text{a}}$	$1.46 \pm 0.70^{\text{a}}$

Note: Different lowercase letters in the same line indicate significant differences ($P < 0.05$).

compounds with significant physiological functions, including cholesterol-lowering and anti-inflammatory effects. The stability of their extraction efficiency is associated with their molecular structural characteristics^[28]. Sterol molecules typically form stable complexes with lipids and exhibit lower sensitivity to enzymatic hydrolysis conditions. The mild reaction conditions of NP are more conducive to preserving the structural integrity of sterols, thereby minimizing potential oxidative losses associated with excessive degradation.

The extraction efficiency of the three key active components—vitamin A, squalene, and total sterols—varied depending on the protease type. This variation stems from the interplay of multiple factors, including the specific action of the enzymes, the influence of reaction conditions on the substrate, and the physicochemical properties of the target components. Consequently, further in-depth studies on blackthroat seaperch liver oil extracted with different proteases are warranted.

3.3 Analysis of fatty acids

Fatty acids, as the core functional components of lipids, possess carbon chain structures, double-bond positions, and saturation levels that directly determine the nutritional properties and physiological functions of oils^[29]. A comparative analysis of the fatty acid composition of oils extracted via the aqueous enzymatic method (PS, TS, NP, and AP) revealed differences in the enrichment of individual fatty acids depending on the protease used (Table 2).

Fatty acid composition indicates that oleic acid constitutes over 50% of the total fatty acid in all four extracts. The highest proportion (53.95%) was observed in the AP extract, indicating a relatively greater contribution of oleic acid to the total fatty acid

composition, despite its lower absolute content, which may be attributed to the efficient dissociation of lipoprotein complexes under alkaline conditions. The strong hydrolytic environment during AP treatment more thoroughly disrupts lipid-protein binding, thereby releasing greater amounts of free oleic acid^[30]. In contrast, the TS exhibited a slightly lower oleic acid proportion (51.88%) in total fatty acid. However, TS demonstrated significantly superior efficacy in releasing long-chain PUFAs, exemplified by its DHA content of 63.65 mg/g, approximately 4.6% higher than that of PS. This advantage is likely due to TS's specific activation of phospholipases^[31]. Its precise peptide bond cleavage capability enables targeted degradation of phospholipid bilayers in cell membranes, facilitating the release of DHA and EPA tightly bound to membrane structures.

The distribution pattern of EPA was more complex, with TS exhibiting the highest content at 21.71 mg/g. Neutral conditions may be more favorable for the hydrolysis of ester bonds at the *sn*-2 position of triglycerides, preferentially releasing EPA bound to medium-chain fatty acids^[32], and the efficient enzymatic hydrolysis of TS releases more EPA but is accompanied by an abnormally high erucic acid content (13.14 mg/g). Linoleic acid was the most abundant (4.95 mg/g) and proportionally abundant in PS, which was associated with its acidic environment activating gastric lipase-acidic conditions promote the hydrolysis of ester bonds at the *sn*-1,3 position, preferentially releasing peripheral linoleic acid^[33]. Arachidonic acid has the highest content (5.88 mg/g) and proportion in total fatty acid (0.77%) in TS, which has significant effects on gonadal development, reproductive performance, lipid metabolism, and steroid hormone synthesis^[34], and acts as a precursor of inflammatory mediators^[35].

Table 2 Fatty acid content (mg/g) of blackthroat seaperch liver oil extracted by different proteases.

Fatty acid	PS	TS	NP	AP
Palmitoleic acid (C _{16:1n-7})	40.35 ± 1.21 ^a	39.76 ± 1.19 ^a	26.52 ± 0.80 ^b	25.66 ± 0.77 ^b
Oleic acid (C _{18:1n-9c})	408.16 ± 12.24 ^a	396.00 ± 11.88 ^a	274.90 ± 8.25 ^b	264.79 ± 7.94 ^b
<i>cis</i> -11-Eicosenoic acid (C _{20:1})	32.79 ± 0.98 ^a	32.36 ± 0.97 ^a	20.07 ± 0.60 ^b	19.59 ± 0.59 ^b
Erucic acid (C _{22:1n-9})	1.49 ± 0.04 ^c	13.14 ± 0.39 ^a	8.26 ± 0.25 ^b	8.28 ± 0.25 ^b
Nervonic acid (C _{24:1n-9})	4.90 ± 0.15 ^a	4.64 ± 0.14 ^{ab}	3.30 ± 0.10 ^{bc}	3.37 ± 0.10 ^c
MUFAs	487.69 ± 14.92 ^a	485.90 ± 14.57 ^a	333.05 ± 10.00 ^b	321.69 ± 9.65 ^b
Linoleic acid (C _{18:2n-6c})	4.95 ± 0.15 ^a	4.57 ± 0.14 ^{ab}	3.66 ± 0.11 ^{bc}	2.86 ± 0.09 ^c
Linolenic acid (C _{18:3n-3})	2.00 ± 0.06 ^a	1.70 ± 0.05 ^b	2.01 ± 0.06 ^a	1.30 ± 0.04 ^c
Arachidonic acid (C _{20:4n-6})	5.74 ± 0.17 ^a	5.88 ± 0.18 ^a	3.45 ± 0.10 ^b	3.24 ± 0.10 ^b
EPA (C _{20:5n-3})	19.57 ± 0.59 ^b	21.71 ± 0.65 ^a	14.25 ± 0.43 ^c	13.22 ± 0.40 ^c
DHA (C _{22:6n-3})	60.86 ± 1.83 ^b	63.65 ± 1.91 ^a	38.31 ± 1.15 ^c	36.55 ± 1.10 ^c
PUFAs	93.12 ± 2.80 ^a	97.51 ± 2.93 ^a	61.68 ± 1.85 ^b	57.17 ± 1.73 ^b
Butyric acid (C _{4:0})	1.82 ± 0.05 ^a	1.47 ± 0.04 ^b	1.81 ± 0.05 ^a	1.44 ± 0.04 ^b
Lauric acid (C _{12:0})	1.82 ± 0.05 ^a	1.41 ± 0.04 ^b	1.83 ± 0.05 ^a	1.76 ± 0.05 ^a
Myristic acid (C _{14:0})	8.64 ± 0.26 ^{ab}	9.17 ± 0.28 ^a	6.54 ± 0.20 ^c	5.66 ± 0.17 ^d
Pentadecanoic acid (C _{15:0})	1.90 ± 0.06 ^a	1.59 ± 0.05 ^b	0.57 ± 0.02 ^c	0.63 ± 0.02 ^c
Palmitic acid (C _{16:0})	119.85 ± 3.60 ^a	117.61 ± 3.53 ^a	81.19 ± 2.44 ^b	73.68 ± 2.21 ^c
Heptadecanoic acid (C _{17:0})	1.94 ± 0.06 ^b	3.54 ± 0.11 ^a	1.75 ± 0.05 ^b	1.82 ± 0.05 ^b
Stearic acid (C _{18:0})	40.90 ± 1.23 ^a	40.58 ± 1.22 ^a	26.93 ± 0.81 ^b	23.20 ± 0.70 ^c
Eicosanoic acid (C _{20:0})	1.02 ± 0.03 ^c	1.25 ± 0.04 ^b	2.17 ± 0.07 ^a	2.12 ± 0.06 ^a
Tetracosanoic acid (C _{24:0})	1.98 ± 0.06 ^b	3.29 ± 0.10 ^a	1.77 ± 0.05 ^b	1.67 ± 0.05 ^b
SFAs	179.87 ± 5.40 ^a	179.91 ± 5.41 ^a	124.56 ± 3.74 ^b	111.98 ± 3.35 ^c

Note: Different lowercase letters in the same line indicate significant differences ($P < 0.05$); EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFAs, saturated fatty acids.

The distribution of SFA exhibited diverse characteristics, with palmitic acid reaching the highest proportion of 15.64% in NP. The mild action of neutral protease may preferentially hydrolyze the peripheral ester bonds of triglycerides, leading to an increase in the proportion of core SFA; the alkaline environment of AP diluted the proportion of palmitic acid, although it increased the total fatty acid extraction. Short-chain fatty acids such as butyric and lauric acids were generally low, but their slightly higher proportions in PS and NP could indicate that acidic or neutral conditions are more favorable for short-chain fatty acid release.

3.4 Analysis of volatile compounds by GC-IMS

The composition of volatile compounds in blackthroat seaperch liver oil extracted by four enzymatic processes was systematically analyzed by GC-IMS. The occurrence of multiple signals for a compound may be attributed to its high proton affinity or dimerization within the drift region. Among the 55 volatiles detected by GC-IMS (Figure 1 and Table S2), aldehydes and ketones were predominant. Long-chain PUFAs were efficiently released by TS and generated aldehydic flavor precursors through oxidative cleavage, which conferred strong grassy and fruity aroma characteristics to the oils^[36], as reflected by the high concentrations of (*E*)-2-pentenal-M and hexanal-M. Meanwhile, the most abundant volatile compounds detected in TS were 1-penten-3-one-D and acetone, suggesting that ketones act as secondary oxidation products to neutralize the irritant properties of aldehydes through subsequent reduction reactions^[37]. In PS, (*E*)-2-pentenal-M and propanal were significantly higher than in the other samples, suggesting that acidic conditions accelerated the oxidation of ω -3 PUFAs to generate a large number of short-chain aldehydes. In addition, SFAs in PS inhibited the generation of short-chain aldehydes such as pentanal-D by enhancing the physical stability of the lipids, but the oxidation products of long-chain aldehydes such as octanal-D still exhibited a potential risk of stale flavor. The

volatiles of NP hydrolysis product were dominated by alcohols and esters of 1-penten-3-ol-M and methyl acetate, suggesting that neutral conditions are more favorable for the reduction and esterification reactions of aldehydes. This property is hypothesized to be closely related to the high proportion of MUFA in NP, where the oxidation pathway of oleic acid tends to generate alcohols rather than irritating aldehydes^[38]. The distribution of volatiles in AP is specific, with thiophene and (1-methylethyl) benzene generate heterocyclic compounds with smoky and burnt aromas. AP hydrolysis product had the highest vitamin A content and significantly inhibited PUFA oxidation, thus hexanal-M and pentanal-D, both the lowest of the four groups, but the alkaline environment may have partially offset some of the antioxidant effects by promoting sulfur metabolic pathways^[39].

The high-resolution chemical fingerprints constructed by GC-IMS can clearly show the changes of volatile compounds, but some volatile compounds cannot be detected due to the limited coverage of the IMS standard database. Therefore, the volatile compounds of different enzymatically extracted blackthroat seaperch liver oil were comprehensively analyzed by combining the analytical system and GC-MS, which is more widely applicable.

3.5 Analysis of volatile compounds by GC-MS combined with ROAV

The limited database of GC-IMS was combined with GC-MS using a robust reference database of standards to ensure a comprehensive analysis of volatile flavor differences in blackthroat seaperch liver oil and to reveal the role of different enzymatic processes in modulating the pathways of volatile compound production. Aldehydes and ketones, as the core products of lipid oxidation^[40], showed significant differences among the different enzymatic processes. As shown in Table 3, the benzaldehyde was detected only in TS, and its bitter almond aroma may be related to the generation

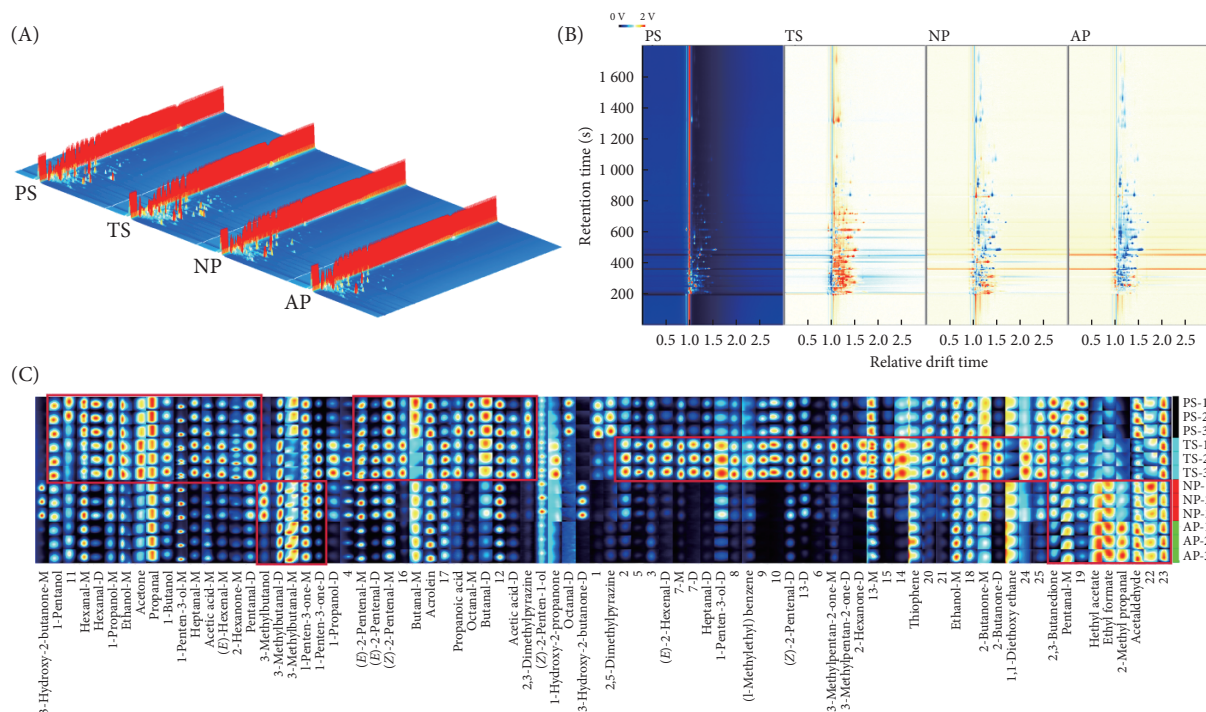


Figure 1 GC-IMS analysis of volatile compounds in blackthroat seaperch liver oil extracted from different proteases. (A) 3D spectra of different samples; (B) 2D spectra of different samples under direct comparison mode; (C) fingerprint comparisons of volatile compounds. M and D represent the monomer and dimer of the corresponding compound, respectively. The red-boxed regions highlight the differences in volatile profiles among the four groups. 1–25, undetermined substances.

Table 3 Volatile compounds of blackthroat seaperch liver oil extracted by different proteases by GC-MS.

Compound	Molecule formula	Retention time (min)	CAS	Relative content (%)			
				PS	TS	NP	AP
Hydrocarbons							
2,2,4,4,6,8,8-Heptamethyl-nonane	C ₁₆ H ₃₄	0.35	4390-4-9	0.34	0.37	0.35	ND
1,4-Dimethyl-bicyclo[2.1.0]pentane	C ₇ H ₁₂	9.53	17065-18-8	4.97	ND	ND	ND
2,3,5,8-Tetramethyl-decane	C ₁₄ H ₃₀	11.12	192823-15-7	ND	ND	ND	2.49
(1-Methylethylidene)-cyclobutane	C ₇ H ₁₂	11.92	1528-22-9	ND	ND	ND	0.88
3-Ethenyl-cyclooctene	C ₁₀ H ₁₆	11.94	2213-60-7	ND	1.99	ND	ND
Spiro[4.5]dec-1-ene	C ₁₀ H ₁₆	12.19	697-27-8	ND	ND	0.67	0.22
3-Prop-2-enylcyclohexene	C ₉ H ₁₄	12.20	15232-95-8	ND	0.61	ND	ND
3-Methyl-4-methylene-hexane	C ₈ H ₁₆	14.11	3404-67-9	ND	0.86	ND	ND
Dodecane	C ₁₂ H ₂₆	15.22	112-40-3	ND	0.55	3.97	2.10
1,3-Bis(1,1-dimethylethyl)-benzene	C ₁₄ H ₂₂	16.79	1014-60-4	ND	0.52	5.68	4.46
2,3,8-Trimethyl-decane	C ₁₃ H ₂₈	17.01	62238-14-6	ND	ND	1.78	ND
4,6-Dimethyl-dodecane	C ₁₄ H ₃₀	17.46	61141-72-8	ND	ND	2.27	ND
5-(3-Buten-1-yl)-1,3-cyclohexadiene	C ₁₀ H ₁₄	17.77	197523-62-9	ND	0.94	ND	ND
α-Pinene	C ₁₀ H ₁₆	17.77	80-56-8	ND	ND	ND	2.18
3,6-Dimethyl-decane	C ₁₂ H ₂₆	17.98	17312-53-7	ND	ND	1.56	ND
Ethylbenzene	C ₈ H ₁₀	18.49	100-41-4	0.11	ND	ND	ND
3-(1-Methyl-2-propenyl)-1,5-cyclooctadiene	C ₁₂ H ₁₈	20.43	16538-88-8	0.28	ND	ND	ND
3-Allyl-1-cyclohexene	C ₉ H ₁₄	20.54	13511-13-2	ND	0.56	ND	ND
5-Methyl-5-propyl-nonane	C ₁₃ H ₂₈	20.61	17312-75-3	ND	0.66	ND	ND
Tetradecane	C ₁₄ H ₃₀	20.61	629-59-4	ND	ND	2.87	ND
2,2-Dimethyl-butane	C ₆ H ₁₄	20.63	75-83-2	ND	ND	ND	0.37
3-Methyl-5-propyl-nonane	C ₁₃ H ₂₈	22.18	31081-18-2	ND	0.39	1.27	0.84
2,6,6-Trimethyl-octane	C ₁₁ H ₂₄	22.20	54166-32-4	ND	ND	0.68	ND
5-Ethyl-cyclohexadiene-1,3-diene	C ₈ H ₁₂	22.44	40085-08-3	0.17	ND	ND	2.69
Pentadecane	C ₁₅ H ₃₂	23.11	629-62-9	0.59	ND	ND	ND
3,3-Dimethyl-hexane	C ₈ H ₁₈	23.11	563-16-6	ND	0.59	ND	ND
3,3-Dimethyl-pentane	C ₇ H ₁₆	23.11	562-49-2	ND	0.23	ND	ND
3-Methyl-heneicosane	C ₂₂ H ₄₆	23.13	6418-47-9	ND	0.65	ND	ND
1,2,4-Trimethyl-benzene	C ₉ H ₁₂	23.61	95-63-6	0.28	ND	ND	ND
2,2-Dimethyl-pentane	C ₇ H ₁₆	27.87	590-35-2	ND	7.69	ND	ND
2,6,10,14-Tetramethyl-pentadecane	C ₁₉ H ₄₀	27.88	1921-70-6	16.09	20.88	33.71	27.16
Total content				22.82	37.49	54.80	43.01
Aldehydes							
Benzaldehyde	C ₇ H ₆ O	8.41	100-52-7	ND	5.34	ND	ND
(E,E)-2,4-Heptadienal	C ₇ H ₁₀ O	9.77	4313-3-5	1.87	ND	ND	ND
Benzeneacetaldehyde	C ₈ H ₈ O	10.76	122-78-1	ND	ND	4.09	ND
Nonanal	C ₉ H ₁₈ O	12.49	124-19-6	2.39	4.85	1.87	4.83
(E)-2-Nonenal	C ₉ H ₁₆ O	14.10	18829-56-6	0.96	ND	ND	ND
3-Ethyl-benzaldehyde	C ₉ H ₁₀ O	14.24	34246-54-3	ND	1.14	ND	ND
Decanal	C ₁₀ H ₂₀ O	15.40	112-31-2	0.28	ND	ND	ND
(E)-2-Decenal	C ₁₀ H ₁₈ O	16.97	3913-81-3	3.04	4.26	ND	2.71
(E,E)-2,4-Decadienal	C ₁₀ H ₁₆ O	17.85	25152-84-5	1.66	ND	ND	ND
Undecanal	C ₁₁ H ₂₂ O	18.21	112-44-7	1.11	1.16	ND	ND
2-Undecenal	C ₁₁ H ₂₀ O	19.71	2463-77-6	10.59	6.49	ND	ND
(E)-Hexadec-2-enal	C ₁₆ H ₃₀ O	19.71	22644-96-8	ND	ND	ND	2.88
Total content				21.90	23.24	5.96	10.42
Ketones							
Furaneol	C ₆ H ₈ O ₃	7.78	3658-77-3	ND	2.40	ND	ND

Table 3 (Continued)

Compound	Molecule formula	Retention time (min)	CAS	Relative content (%)			
				PS	TS	NP	AP
2-Oxo-2-phenylethyl formate	C ₉ H ₈ O ₃	8.45	55153-12-3	ND	ND	3.04	ND
5-Hydroxy-7-methoxy-2-methyl-3-phenyl-4-chromenone	C ₁₇ H ₁₄ O ₄	9.44	55927-39-4	ND	ND	2.39	ND
1,4-Cyclohexanedione	C ₆ H ₈ O ₂	10.36	637-88-7	1.68	0.99	ND	ND
(R)-2-(1,1-Dimethylethyl)-6-methyl-4H-1,3-dioxin-4-one	C ₉ H ₁₄ O ₃	11.10	107289-20-3	ND	ND	3.04	ND
3,5-Octadien-2-one	C ₈ H ₁₂ O	11.53	38284-27-4	0.37	0.49	ND	1.72
(E,E)-3,5-Octadien-2-one	C ₈ H ₁₂ O	12.19	30086-02-3	1.75	ND	ND	ND
2,3-Octanedione	C ₈ H ₁₄ O ₂	12.49	585-25-1	ND	ND	ND	0.46
4-Hydroxy-3,5,5-trimethylcyclohex-2-enone	C ₉ H ₁₄ O ₂	15.63	14203-59-9	ND	ND	0.49	ND
3,4-Dihydroxyacetophenone	C ₈ H ₈ O ₃	15.88	1197-09-7	0.79	ND	ND	ND
Methyl vinyl ketone	C ₄ H ₆ O	16.96	78-94-4	ND	0.91	ND	ND
7-Ethyl-4-nonanone,	C ₁₁ H ₂₂ O	17.46	40237-88-5	1.88	ND	ND	ND
(E)-8-Methyl-3,7-nonadien-2-one	C ₁₀ H ₁₆ O	17.46	35408-14-1	ND	ND	ND	0.51
2-Methyl-3-decanone	C ₁₁ H ₂₂ O	17.47	5463-82-1	ND	ND	ND	0.42
2-Undecanone	C ₁₁ H ₂₂ O	17.85	112-12-9	ND	1.28	ND	2.10
Ortho-hydroxypropiophenone	C ₉ H ₁₀ O ₂	18.60	610-99-1	1.01	ND	ND	ND
3-Ethyl-3-nonen-2-one	C ₁₁ H ₂₀ O	19.14	56312-56-2	ND	ND	ND	0.33
(2E)-1-(3-Furanyl)-4-methyl-2-penten-1-one	C ₁₀ H ₁₂ O ₂	19.65	34348-59-9	ND	2.84	ND	ND
2-Octen-4-one	C ₈ H ₁₄ O	19.72	4643-27-0	ND	0.99	ND	ND
3-Nonen-5-one	C ₉ H ₁₆ O	20.34	82456-34-6	0.33	ND	ND	ND
4-(2,6,6-Trimethyl-1-cyclohexen-1-yl)-3-buten-2-one	C ₁₃ H ₂₀ O	22.88	14901-07-6	ND	ND	ND	1.62
<i>trans</i> -β-Ionone	C ₁₃ H ₂₀ O	22.89	79-77-6	2.40	2.71	0.56	1.51
3',5'-Dihydroxyacetophenone	C ₈ H ₈ O ₃	24.01	51863-60-6	ND	0.33	ND	0.49
1-Ethyl-4-piperidone	C ₇ H ₁₃ NO	27.88	3612-18-8	ND	ND	6.60	ND
Total content				10.22	12.94	16.11	9.16
Alcohols							
Pipradrol	C ₁₈ H ₂₁ NO	9.53	467-60-7	1.52	ND	ND	ND
2,6-Dimethyl-cyclohexanol	C ₈ H ₁₆ O	12.64	5337-72-4	1.42	2.90	4.15	4.43
2-Isopropenyl-5-methyl-6-hepten-1-ol	C ₁₁ H ₂₀ O	15.85	13066-55-2	3.08	ND	ND	ND
<i>endo</i> -Borneol	C ₁₀ H ₁₈ O	16.30	507-70-0	1.03	ND	ND	ND
<i>cis</i> -1,3-Dimethyl-cyclohexanol	C ₈ H ₁₆ O	16.31	15466-94-1	ND	1.95	ND	2.21
1-Methylcycloheptanol	C ₈ H ₁₆ O	16.31	3761-94-2	ND	ND	ND	1.11
3,3,6-Trimethyl-1,5-heptadien-4-ol	C ₁₀ H ₁₈ O	16.60	27644-04-8	1.17	ND	ND	ND
2-(1-Methylethyl)-phenol	C ₉ H ₁₂ O	17.23	88-69-7	0.25	ND	ND	ND
3,6-Dimethyl-2,6-octadiene-4,5-diol	C ₁₀ H ₁₈ O ₂	17.46	10317-05-2	ND	ND	0.53	ND
1-Methyl-cyclopentanol	C ₆ H ₁₂ O	17.79	1462-03-9	ND	ND	2.81	ND
1,2-Dimethylcyclopentanol	C ₇ H ₁₄ O	17.85	19550-45-9	ND	ND	1.75	ND
(-)-Myrtenol	C ₁₀ H ₁₆ O	17.96	19894-97-4	4.17	2.20	ND	ND
1-Phenyl-2-propanol	C ₉ H ₁₂ O	17.97	14898-87-4	ND	ND	ND	0.27
4-Amino-5,6-dimethoxy-2-cyclohexen-1-enol	C ₈ H ₁₅ NO ₃	18.00	109906-47-0	ND	ND	0.40	ND
Cyclooctyl alcohol	C ₈ H ₁₆ O	18.22	696-71-9	ND	0.83	ND	ND
4-Allyl-1,6-heptadiene-4-ol	C ₁₀ H ₁₆ O	19.13	10202-75-2	ND	0.22	ND	ND
1,3-Benzenediol, 5-pentyl-	C ₁₁ H ₁₆ O ₂	23.83	500-66-3	1.92	1.18	ND	ND
Total content				14.56	9.28	9.65	8.01
Acids							
Propanoic acid, anhydride	C ₆ H ₁₀ O ₃	7.76	123-62-6	ND	ND	2.58	ND
7-Oxabicyclo[2.2.1]hept-5-ene-2,3-dicarboxylic anhydride	C ₈ H ₆ O ₄	9.56	5426-9-5	ND	ND	ND	1.00
1-Amino-cyclohexanecarboxylic acid	C ₇ H ₁₃ NO ₂	12.48	2756-85-6	ND	0.99	ND	0.83

Table 3 (Continued)

Compound	Molecule formula	Retention time (min)	CAS	Relative content (%)			
				PS	TS	NP	AP
<i>cis</i> -4-(Hydroxymethyl)cyclohexanecarboxylic acid	C ₈ H ₁₄ O ₃	12.63	73094-35-6	0.55	ND	ND	ND
Total content				0.55	0.99	2.58	1.84
Esters							
4-Nitrophenylanthranilate	C ₁₃ H ₁₀ N ₂ O ₄	10.75	19176-60-4	0.34	ND	ND	ND
2-Furancarboxylic acid, 2-tetrahydrofurylmethyl ester	C ₁₀ H ₁₂ O ₄	11.11	4623-4-5	ND	ND	ND	1.65
Methanesulfonic acid, ethyl ester	C ₃ H ₈ O ₃ S	11.51	62-50-0	ND	ND	ND	0.26
Ethyl diazoacetate	C ₄ H ₆ N ₂ O ₂	12.48	623-73-4	ND	ND	ND	1.44
Ethaneperoxoic acid, 1-cyano-1-[2-(2-phenyl-1,3-dioxolan-2-yl)ethyl]pentyl ester	C ₁₉ H ₂₅ NO ₅	14.15	58422-92-7	ND	ND	1.83	ND
Methyl 1-methylpiperidine-2-carboxylate	C ₈ H ₁₅ NO ₂	14.53	1690-74-0	ND	0.24	ND	ND
4-Allylphenyl acetate	C ₁₁ H ₁₂ O ₂	16.31	61499-22-7	0.70	ND	ND	ND
(<i>Z</i>)-3-Hexenyl propanoate	C ₉ H ₁₆ O ₂	16.96	33467-74-2	ND	0.89	ND	ND
2-Cyclopentene-1-carboxylic acid, 1,2,3-trimethyl-, ethyl ester	C ₁₁ H ₁₈ O ₂	17.47	29915-95-5	ND	ND	0.46	ND
Benzyl mandelate	C ₁₅ H ₁₄ O ₃	17.49	890-98-2	0.38	ND	ND	ND
4-Methylphenyl β-phenylpropionate	C ₁₆ H ₁₆ O ₂	17.97	22020-95-7	ND	0.59	ND	ND
Cyclopropyl phenylmethanol	C ₁₀ H ₁₂ O	17.97	1007-03-0	ND	ND	ND	3.27
(<i>E</i>)-2-Hexen-1-ol, propanoate	C ₉ H ₁₆ O ₂	18.22	53398-80-4	0.15	ND	ND	ND
Benzoic acid, phenyl ester	C ₁₃ H ₁₀ O ₂	18.49	93-99-2	0.25	ND	ND	ND
Hexadecanoic acid, (2-pentadecyl-1,3-dioxolan-4-yl)methyl ester	C ₃₅ H ₆₈ O ₄	18.70	41563-11-5	ND	0.36	ND	ND
Benzoic acid, 2,5-dimethyl-, (2,4-dimethylphenyl)methyl ester	C ₁₈ H ₂₀ O ₂	22.44	55000-48-1	0.25	ND	ND	ND
(2,6,6-Trimethyl-2-hydroxycyclohexylidene) acetic acid lactone	C ₁₁ H ₁₆ O ₂	23.99	17092-92-1	0.80	ND	ND	ND
Total content				2.87	2.09	2.28	6.63
Ethers							
1-(Hexyloxy)-4-methyl-hexane	C ₁₃ H ₂₈ O	11.14	74421-20-8	ND	1.71	ND	ND
(1-Methoxy-pentyl)-cyclopropane	C ₉ H ₁₈ O	15.85	94883-96-2	0.52	ND	ND	ND
4-Butoxy-benzenamine	C ₁₀ H ₁₅ NO	16.60	4344-55-2	ND	0.67	ND	ND
1,1'-Oxybis-hexane	C ₁₂ H ₂₆ O	18.00	112-58-3	ND	ND	0.56	ND
Benzyl nopyl ether	C ₁₈ H ₂₄ O	22.45	74851-17-5	1.15	ND	ND	ND
2-[(Ethylsulfanyl)methyl]-furan	C ₁₉ H ₄₀	27.88	2024-70-6	ND	ND	1.90	0.76
Total content				1.67	2.38	2.47	0.76
Heterocycles							
1 <i>H</i> -Tetrazol-5-amine	CH ₃ N ₅	7.78	4418-61-5	ND	1.19	ND	ND
2-Pentyl-furan	C ₉ H ₁₄ O	9.26	3777-69-3	2.44	ND	ND	ND
(<i>Z</i>)-2-(1-Pentenyl)-furan	C ₉ H ₁₂ O	9.51	20992-60-3	ND	ND	1.96	ND
(<i>E</i>)-2-(1-Pentenyl)-furan	C ₉ H ₁₂ O	9.51	20992-69-2	ND	ND	ND	7.37
<i>N</i> -3-(Pyridinyl)acetamide	C ₇ H ₈ N ₂ O	9.51	5867-45-8	ND	ND	ND	2.06
<i>cis</i> -2-(2-Pentenyl)furan	C ₉ H ₁₂ O	9.53	70424-13-4	12.11	4.14	ND	ND
2-Propyl-furan	C ₇ H ₁₀ O	9.82	4229-91-8	ND	ND	ND	3.12
2,3-Dimethyl-pyrazine	C ₆ H ₈ O ₂	10.37	5910-89-4	0.16	ND	ND	ND
1,2,4-Triazolo[4,3- <i>b</i>]pyridazine	C ₅ H ₄ N ₄	10.75	274-83-9	ND	ND	0.59	ND
Furan-2-carboxylic acid benzoyloxyamide	C ₁₂ H ₉ NO ₄	11.53	59101-38-1	ND	ND	2.22	ND
1-(2-Furanyl)-1-propanone	C ₇ H ₈ O ₂	11.54	3194-15-8	ND	ND	ND	1.19
Furan	C ₄ H ₄ O	11.75	110-00-9	ND	ND	ND	0.65
Pentaleno[1,2- <i>b</i>]oxirene, octahydro-, (1 <i>a</i> . <i>α</i> .,1 <i>b</i> . <i>α</i> .,4 <i>a</i> . <i>β</i> .,5 <i>a</i> . <i>α</i> .)-	C ₈ H ₁₂ O	12.19	55449-71-3	ND	1.70	ND	ND
2(1 <i>H</i>)-Pyridinone	C ₅ H ₅ NO	12.50	142-08-5	ND	0.54	ND	ND
4,5-Dimethyl-thiazole	C ₅ H ₇ NS	16.32	3581-91-7	0.12	ND	ND	ND

Table 3 (Continued)

Compound	Molecule formula	Retention time (min)	CAS	Relative content (%)			
				PS	TS	NP	AP
(Z)-1-Amino-4,4-dimethyl-1-pyridin-3-yl-pent-1-en-3-one	C ₁₂ H ₁₆ N ₂ O	16.78	136346-90-2	ND	ND	0.71	ND
1-Methyl-7-hydroxy-6-methoxy-3,4-dihydroisoquinolin	C ₁₁ H ₁₃ NO ₂	16.79	4602-71-5	ND	ND	ND	0.78
1H-Pyrrole-3-carbonitrile	C ₅ H ₄ N ₂	17.77	7126-38-7	1.27	ND	ND	ND
(1-Methylethenyl)-pyrazine	C ₇ H ₈ N ₂	17.96	38713-41-6	1.10	ND	ND	ND
Dicyclopentadiene diepoxide	C ₁₀ H ₁₂ O ₂	18.48	81-21-0	1.94	ND	ND	ND
2-Hexyl-furan	C ₁₀ H ₁₆ O	18.48	3777-70-6	ND	1.49	ND	ND
α-Oxo-furan-2-acetonitrile	C ₆ H ₃ NO ₂	19.71	6047-91-2	ND	1.41	ND	ND
2,3,4a,4b,8a,8b-Hexahydrobenzo[3,4]cyclobuta[1,2-b]-1,4-dioxin	C ₁₀ H ₁₂ O ₂	19.76	69956-61-2	0.86	ND	ND	ND
5-Heptyl-pyrrolidin-2-one	C ₁₁ H ₂₁ NO	27.88	2405-92-7	3.77	ND	ND	ND
Total content				23.74	10.47	5.48	14.52
Amines							
N,N-Dimethyl-1-propanamine	C ₅ H ₁₃ N	12.12	926-63-6	ND	0.27	ND	ND
3,7-Dimethyl-1,7-octadien-3-amine	C ₁₀ H ₁₉ N	12.49	77984-56-6	ND	ND	ND	0.60
N-Ethyl-N-(but-3-enyl)pentan-3-amine	C ₁₁ H ₂₃ N	12.62	89214-96-0	ND	ND	ND	0.58
2-(2-Methoxyphenyl)ethylamine	C ₉ H ₁₃ NO	14.59	2045-79-6	0.20	ND	ND	ND
Cyclohexylamine	C ₆ H ₁₃ N	15.23	108-91-8	ND	ND	0.45	ND
5-(2-Aminoethyl)-1,3-benzodioxole hydrochloride	C ₉ H ₁₁ NO ₂	17.52	1653-64-1	ND	0.38	ND	ND
3-(Diethylamino)-propanenitrile	C ₇ H ₁₄ N ₂	20.14	5351-4-2	0.76	ND	ND	ND
Total content				0.97	0.65	0.45	1.17
Others							
N,N'-Ethylenebis-acrylamide	C ₈ H ₁₂ N ₂ O ₂	10.34	2956-58-3	ND	0.48	ND	ND
2-Isopropenyl-1-methylcyclobutane acetonitrile	C ₁₀ H ₁₅ N	12.47	55760-15-1	ND	ND	ND	0.50
Dicyandiamide	C ₂ H ₄ N ₄	12.63	461-58-5	ND	ND	0.22	ND
4-Propylbenzaldehyde diethyl acetal	C ₁₄ H ₂₂ O ₂	22.89	89557-35-7	0.70	ND	ND	ND
3-Heptylthiophene	C ₁₁ H ₁₈ S	27.87	65016-61-7	ND	ND	ND	3.47
Total content				0.70	0.48	0.22	3.97

Note: ND indicates data not detected.

of phenylalanine released by TS hydrolysis. In contrast, benzaldehyde was not detected by GC-IMS, which may be attributed to the higher sensitivity of GC-MS for the detection of aromatic aldehyde compounds. (*E*)-2-Decenal and (*E,E*)-2,4-heptadienal, were detected in PS, together with the high signal of propionaldehyde in GC-IMS, contributing to the pungent and grassy aroma characteristics of this group of fats. The detection of long-chain unsaturated aldehydes, such as (*E*)-hexadec-2-enal, in AP, may be the result of alkaline conditions that promoted the progression of lipid oxidation and the formation of more complex, high-molecular-weight flavor substances.

The composition of hydrocarbons, which accounted for 22.82%–54.80% of the total volatile components, suggests the specific action of enzymatic processes on the lipid structure. The faint waxy aroma of 2,6,10,14-tetramethyl-pentadecane, in TS may originate from the highly efficient hydrolysis of lipoprotein complexes by TS, whereas the enrichment in NP hydrolysis product of tetradecane and 4,6-dimethyl-dodecane enrichment in NP reflects the mild oxidation state of lipids under neutral conditions. α-Pinene was detected in AP hydrolysis product, along with (1-methylpropyl)-benzene in GC-IMS, suggesting that alkaline environments promote the generation of branched-chain alkanes

and aromatic hydrocarbons. However, most alkanes usually have high odor thresholds and have little effect on aquatic volatile compounds.

The highest content of esters was found in AP hydrolysis product, among which cyclopropyl phenylmethanol and 2-furancarboxylic acid, 2-tetrahydrofurylmethyl ester were the specific components detected by GC-MS, which showed its advantage in the detection of high molecular weight esters. Heterocyclic compounds, as key contributors to flavor complexity, were expressed in TS hydrolysis product as toasted aroma of *cis*-2-(2-pentenyl)furan and nutty aroma of 2-hexyl-furan, 5-heptyl-pyrrolidin-2-one's cheesy aroma in PS hydrolysis product together with the high concentration of acetic acid detected by GC-IMS exacerbated the acidic flavor perception, reflecting the complexity of lipid-protein interactions under acidic conditions^[41]. The high content of (*E*)-2-(1-pentenyl)-furan in AP hydrolysis product and the sulphide aroma of thiophene by GC-IMS constitute a distinctive smoky flavor that may originate from the pathway by which alkaline environments promote the degradation of sulfur-containing amino acids.

The flavor profiles of blackthroat seaperch liver oil extracted by different enzymatic methods were analyzed by integrating the

ROAVs, as shown in Table 4. In PS group, (*E*)-2-nonenal, with an ROAV of 100, was highly dominant; its very low olfactory threshold meant that even trace amounts contributed significantly to the grassy aroma and fat oxidation flavor. This result, together with the high concentrations of propionaldehyde and (*E*)-2-pentenal detected by GC-IMS, may explain the pronounced pungent flavor of PS hydrolysis product. The complexity of the oxidized flavor was further enhanced by the contribution of (*E,E*)-2,4-decadienal and nonanal. Furaneol was the dominant aroma component in the TS group with an ROAV of 100. Despite the low concentration of furaneol, its very low olfactory threshold made it far superior to the other compounds in terms of sensory contribution. Nonanal and (*E*)-2-decenal added layers of flavor through the grassy and fruity aroma, while the almond aroma of 3-ethyl-benzaldehyde could be attributed to the degradation of phenylalanine released by TS hydrolysis to produce aromatic aldehydes^[42]. NP hydrolysis product exhibited different flavor characteristics. Benzeneacetaldehyde was the core aroma substance with an ROAV of 100. Neutral conditions may promote the conversion of aldehydes to alcohols, such as 1-penten-3-ol and ethanol as detected by GC-IMS, by inhibiting lipid overoxidation. AP was the most distinctive in terms of flavor profile. 4-(2,6,6-Trimethyl-1-cyclohexen-1-yl)-3-buten-2-one with an ROAV of 100 occupied the dominance. The alkaline environment may reduce the

production of undesirable odors such as hexanal by promoting the degradation of sulfur-containing amino acids, thereby generating thiophene and its derivatives, while also inhibiting the oxidation of *n*-6 PUFA.

The integration of GC-MS and GC-IMS provided a comprehensive characterization of the volatile compound profile. High-boiling compounds detected by GC-MS were not detected in GC-IMS and were confirmed to be key aroma components by ROAV analysis; low molecular weight aldehydes detected by GC-IMS, although relatively low in GC-MS, were revealed to have a potential impact on the flavor by their high ROAV, and the combination of the two techniques provided a multi-dimensional perspective for comprehensively analyzing the flavor composition.

3.6 Oxidative stability analysis by Rancimat method

The oxidative stability of blackthroat seaperch liver oil was determined by the Rancimat method as shown in Figure 2A, and the oxidation induction times of different fish oils were in the order of PS > AP > TS > NP, and this result was closely related to its fatty acid composition and the characteristics of the enzymatic process. PS group showed optimal antioxidant capacity, which was mainly attributed to its high percentage of oleic acid of 52.94% and the highest squalene content. The low number of double bonds and

Table 4 ROAV of volatile compounds in blackthroat seaperch liver oil extracted by different proteases.

Compound	Olfactory threshold ($\mu\text{g/kg}$)	ROAV			
		PS	TS	NP	AP
(<i>E</i>)-2-Nonenal	0.001	100.000	ND	ND	ND
(<i>E,E</i>)-2,4-Decadienal	0.002	82.631	ND	ND	ND
2-Pentyl-furan	0.020	14.679	ND	ND	ND
Nonanal	0.020	13.648	20.221	3.875	17.871
Decanal	0.003	12.447	ND	ND	ND
(<i>E</i>)-2-Decenal	0.077	4.507	4.618	ND	2.609
<i>endo</i> -Borneol	0.050	2.265	ND	ND	ND
(<i>E,E</i>)-3,5-Octadien-2-one	0.100	1.999	ND	ND	ND
Undecanal	0.140	0.905	0.692	ND	ND
1,2,4-Trimethyl-benzene	0.590	0.054	ND	ND	ND
2-(1-Methylethyl)-phenol	1.000	0.029	ND	ND	ND
4,5-Dimethyl-thiazole	0.470	0.028	ND	ND	ND
2,3-Dimethyl-pyrazine	0.880	0.020	ND	ND	ND
Ethylbenzene	0.730	0.018	ND	ND	ND
Furaneol	0.002	ND	100.000	ND	ND
3-Ethyl-benzaldehyde	0.085	ND	1.120	ND	ND
2-Octen-4-one	0.200	ND	0.415	ND	ND
Methyl vinyl ketone	0.500	ND	0.152	ND	ND
Dodecane	0.770	ND	0.060	0.214	0.201
2-Undecanone	3.000	ND	0.036	ND	0.052
Cyclooctyl alcohol	10.000	ND	0.007	ND	ND
2,2-Dimethyl-pentane	156	ND	0.004	ND	ND
Benzeneacetaldehyde	0.001 7	ND	ND	100.000	ND
Propanoic acid, anhydride	3.400	ND	ND	0.032	ND
4,6-Dimethyl-dodecane	5.000	ND	ND	0.019	ND
Cyclohexylamine	9.830	ND	ND	0.002	ND
4-(2,6,6-Trimethyl-1-cyclohexen-1-yl)-3-buten-2-one	0.001	ND	ND	ND	100.000
α -Pinene	0.100	ND	ND	ND	1.611
Furan	28.000	ND	ND	ND	0.002

Note: ND indicates data not detected.

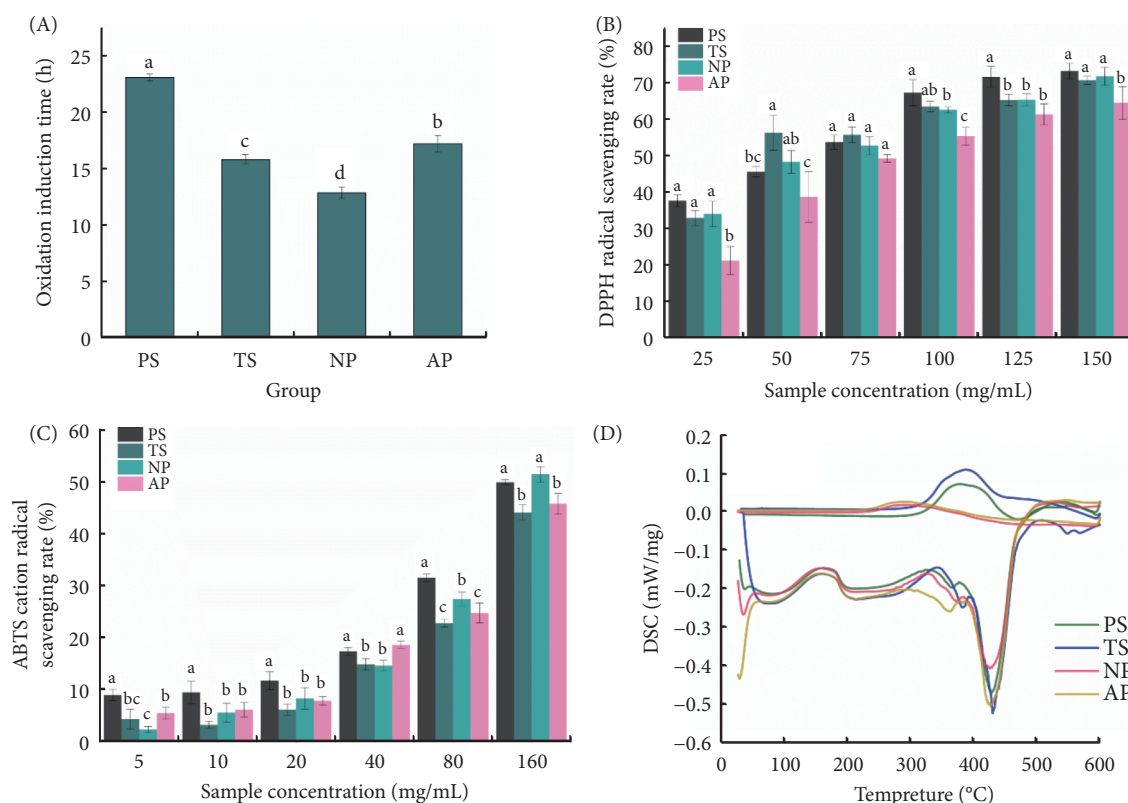


Figure 2 Effects of different extraction methods on the properties of blackthroat seaperch liver oil. (A) Oxidation stability; (B) DPPH radical and (C) ABTS cation radical scavenging abilities; (D) thermal properties. Different lowercase letters indicate significant differences between groups ($P < 0.05$).

stable structure of oleic acid reduce oxidative sensitivity, and squalene, as a natural antioxidant, slows down the oxidation process by scavenging free radicals and blocking the lipid peroxidation chain reaction^[43]. The highest proportion of oleic acid in AP group creates a synergistic effect with the vitamin A content: oleic acid stabilizes the lipid matrix and reduces oxygen penetration through hydrophobicity, while vitamin A directly neutralizes free radicals. At the same time, alkaline conditions may destroy the structure of some natural antioxidant components, resulting in a partial loss of their antioxidant efficacy. The relatively low oxidative stability of TS hydrolysis product may be associated with its high content of ω -3 PUFA, which contain multiple double bonds and are highly susceptible to autoxidation, resulting in the formation of hydroperoxides. The shortest induction time of NP is related to the highest proportion of palmitic acid in its fatty acid composition. Palmitic acid as a SFA itself is more stable, but the higher proportions of linoleic acid and DHA in NP hydrolysis product dominated the oxidation process. Rancimat analyses indicated that the oxidative stability of blackthroat seaperch liver oil not only depended on the fatty acid composition, but was also regulated by a combination of enzymatic conditions and the synergistic effect of bioactive components, so further analysis of the key oxidative stability-related components of blackthroat seaperch liver oil extracted by different enzymes is needed.

3.7 Analysis of DPPH radical and ABTS cation radical scavenging capacity

The antioxidant activities of all four enzymatic products were found to be concentration-dependent based on the DPPH radical

scavenging assay (Figure 2B). The scavenging rate of the PS group increased from 37.89% to 73.17% within the concentration range of 25–150 mg/mL, reflecting excellent stability. Oleic acid and squalene from the PS group form a synergistic mechanism: the monounsaturated structure of oleic acid jointly enhances antioxidant efficacy by decreasing lipid oxidation susceptibility, whereas squalene works by direct scavenging of free radicals and blocking the lipid peroxidation chain reaction^[44]. The TS group reached 70.65% clearance at 150 mg/mL, but the initial value at low concentration (25 mg/mL) was only 32.51%. Although its DHA and EPA had high oxidative risk, vitamin A played a dominant role by neutralizing free radicals at high concentration. The scavenging rate of 71.71% in the NP group at 150 mg/mL was attributed to PUFA as the main oxidized substrate. Sterols may complex with metal ions under neutral conditions resulting in fluctuating data. The AP group had the lowest scavenging rate (21.15%) at low concentration (25 mg/mL) but elevated to 64.07% at high concentration (150 mg/mL). Vitamin A synergizes with oleic acid to enhance antioxidant efficacy but alkaline conditions may damage vitamin A.

The antioxidant activity of blackthroat seaperch liver oil was assessed by ABTS cation radical scavenging assay (Figure 2C), and the scavenging rate of the PS group reached 49.96% at 160 mg/mL, with a fluctuation of $(8.92 \pm 7.22)\%$ at its low concentration (5 mg/mL) originating from the stage-specific effect of acidic conditions on squalene release. Of interest was the sudden increase in clearance to 31.55% at 80 mg/mL in the PS group, which may be related to the squalene release threshold. The TS group was elevated to 44.12% at 160 mg/mL, confirming the cumulative effect of vitamin A. The NP group reached 51.50% at 160 mg/mL, which

may result from palmitic acid stabilization combined with the antagonistic effect of PUFA to affect its activity. The increase in AP group from 5.43% at 5 mg/mL to 45.83% at 160 mg/mL was attributed to the synergistic effect of oleic acid and vitamin A synergism intensified by the alkaline environment. Different proteases affect the antioxidant properties of blackthroat seaperch liver oil by specifically altering the fatty acid composition and antioxidant content. The four groups of samples showed different concentration-dependent response patterns: PS and AP groups showed similar scavenging efficacy at high concentrations, but PS group exhibited a more stable response, while TS and NP groups required relatively high concentrations to achieve comparable scavenging levels.

3.8 Thermal stability analysis

The heat flow-temperature curves of the four enzymatically treated blackthroat seaperch liver oils, as shown in Figure 2D, exhibited dynamic thermal responses that were closely related to their fatty acid compositions and volatile compounds, as determined by DSC analysis. The differences in the morphology of the heat flow curves indicated that the different proteases used for enzymatic treatment affected the thermal stability and phase transition behavior of blackthroat seaperch liver oil. During the initial heating stage (0–200 °C), the green curve of PS first exhibited a broad gradual endothermic valley in the 30–80 °C interval, with the lowest heat flow value dropping to -0.23 mW/mg, followed by the formation of a second shallow endothermic peak of -0.17 mW/mg near 120 °C. The multistage melting behavior is closely related to its high proportion of unsaturated fatty acids^[45].

When the temperature was increased to 200–400 °C, the heat flow curves of all the samples entered a stage of violent oscillation, with the most significant fluctuations in PS and TS groups. The PS group showed a gradual increase in heat flow at approximately 250 °C, followed by an exothermic pulse at 320 °C (-0.16 mW/mg), and this bidirectional change in heat flow may be associated with thermal reactions involving ω -3 fatty acids, including oxidation and possible degradation processes. The six double bonds in DHA make it highly susceptible to thermal oxidation reactions at high temperatures. The initial endothermic event may be associated with energy absorption during the disruption of unsaturated lipid structures, whereas the subsequent exothermic event may be related to further oxidative reactions and structural transformations of oxidation products. These thermal reactions may contribute to the formation of volatile compounds associated with the characteristic fishy odor of fish liver oil. It is worth noting that the intensities of the exothermic peaks of TS group were lower than those of PS group in this temperature region, which may be associated with differences in the thermal reaction behaviors of PUFAs and their potential contributions to flavor precursor formation^[46]. In contrast, the curves of NP and AP groups fluctuated more gently, and no obvious exothermic signals were observed in the 300–350 °C interval. Entering the pyrolysis stage (400–600 °C), the heat flow curves of PS and TS groups highlighted sharp heat absorption valleys in the range of 420–450 °C, with PS group reaching a minimum of -0.51 mW/mg, which corresponded to the cleavage of glycerol and the carbonization of long-chain fatty acids. The concentration of nitrogen-containing heterocyclic compounds (e.g., pyridines and pyrazines) released from PS group in this temperature region was higher than that of the other samples.

During the cooling stage, PS exhibited two distinct exothermic

responses in the 200–280 °C range, with peak intensities of approximately 0.12 and 0.08 mW/mg. They may reflect thermal responses associated with thermally transformed lipid-derived materials generated during the preceding high-temperature treatment. It should be noted that the 600 °C upper temperature limit was used solely as an extreme analytical thermal simulation to characterize the extended thermal response of the liver oils under accelerated conditions and does not represent the temperatures typically encountered during the processing, storage, or handling of fish oils. Furthermore, these high-temperature DSC results are subject to certain limitations, as the use of aluminum crucibles and substantial thermal degradation of the lipid matrix may affect the measured heat-flow signals and increase uncertainty in the assignment of individual thermal events.

Combining the dynamic characteristics of the heat flow curves and the chemical composition data, the effect of enzymatic digestion on the flavor of blackthroat seaperch liver oil may involve a dual regulatory mechanism: PS and TS selectively hydrolyze hydrophobic proteins, releasing PUFA-enriched lipid fractions, which are highly reactive fatty acids that generate rich flavor precursors during heat treatment, but also pose a risk of off-flavor due to oxidative susceptibility. On the other hand, neutral and alkaline proteases may weaken the amplitude of heat flux oscillations at high temperatures by retaining more SFAs and promoting the formation of lipid-peptide affixes, thus enhancing the oxidative stability of fats and oils while losing part of the flavor complexity.

4 Conclusions

The aqueous enzymatic extraction method achieved significantly higher oil yields than solvent extraction, with TS showing the highest extraction efficiency. Enzyme selection markedly influenced the nutritional composition of blackthroat seaperch liver oil: AP favored vitamin A enrichment, PS promoted squalene extraction, and NP was more suitable for total sterol recovery. TS released higher levels of DHA and EPA but also resulted in an unusually high accumulation of erucic acid, whereas AP increased oleic acid content and NP maintained a more balanced fatty acid profile. GC-MS and GC-IMS analyses demonstrated that enzyme type significantly influenced flavor formation. TS promoted PUFA oxidation and aldehyde generation, PS accelerated the formation of short-chain aldehydes, NP produced a milder flavor profile dominated by alcohols and esters, and AP contributed smoky notes through thiol metabolism. Antioxidant activities increased in a concentration-dependent manner, with PS showing greater stability in DPPH radical and ABTS cation radical scavenging activities, while TS and NP exhibited relatively high scavenging capacities at higher concentrations. DSC analysis further revealed that thermal behavior was closely associated with flavor release. The thermal flow fluctuations observed in TS and PS groups during the mid-temperature range reflect the thermal decomposition of ω -3 fatty acids into fishy odor precursors. Due to their high SFAs content, NP and AP exhibit gradual thermal decomposition, which suppresses off-flavors but results in a relatively monotonous flavor profile. Overall, enzyme selection plays a critical role in determining the yield, nutritional quality, flavor characteristics, antioxidant activity, and thermal properties of blackthroat seaperch liver oil.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Author contributions

Jixiang Zhang: Writing-original draft, data curation and formal analysis. Feifan Wu: Data curation and methodology. Chenyang Zhao: Data curation and formal analysis. Xiaoguo Ying: Conceptualization, methodology, project administration and writing-review & editing. Shanggui Deng: Supervision. Wenxuan Chen: Resources and supervision. Alessandra Guidi: Validation. Lukai Ma: Writing-original draft, visualization, formal analysis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at <http://doi.org/10.26599/FSAP.2026.9240185>.

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