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Research article

Comprehensive biochemical analysis and nutritional evaluation of fatty acid and amino acid profiles in eight seahorse species (*Hippocampus* spp.)

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ABSTRACT

Seahorses are increasingly recognized for their nutritional potential, which underscores the necessity for comprehensive biochemical analyses. This study aims to investigate the fatty acid and amino acid compositions of eight seahorse species, including both genders of Hippocampus trimaculatus, Hippocampus kelloggi, Hippocampus abdominalis, and *Hippocampus erectus*, to evaluate their nutritional value. We employed Gas Chromatography-Mass Spectrometry (GC-MS) and High-Performance Liquid Chromatography (HPLC) to analyze the fatty acid and amino acid profiles of the seahorse species. GC-MS was used to detect 34 fatty acid methyl esters, while HPLC provided detailed amino acid profiles. GC-MS analysis demonstrated high precision with relative standard deviations (RSDs) generally below 2.53 %, satisfactory repeatability (RSDs from 6.55 % to 8.73 %), and stability (RSDs below 2.82 %). Recovery rates for major fatty acids ranged from 98.73 % to 109.12 %. HPLC analysis showed strong separation of amino acid profiles with theoretical plate numbers exceeding 5000. Precision tests yielded RSDs below 1.23 %, with reproducibility and stability tests showing RSDs below 2.73 % and 2.86 %, respectively. Amino acid recovery rates ranged from 97.58 % to 104.66 %. Nutritional analysis revealed significant variations in fatty acid content among the species. Female *H. erectus* showed higher levels of hexadecanoic acid and saturated fatty acids, while male *H. abdominalis* had lower concentrations of n-3 full cis 4,7,10,13,16,19-docosahexaenoic acid (DHA). Total lipid yields varied from 3.2491 % to 12.3175 %, with major fatty acids constituting 17.9717 %–74.6962 % of total lipids. In

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conclusion, this study provides essential insights into the fatty acid and amino acid composition of seahorses, supporting their potential as valuable dietary supplements. The differences between genders in specific fatty acids suggest a nuanced nutritional profile that could be exploited for targeted dietary applications. Further research is needed to explore the seasonal and environmental variations affecting seahorse biochemical composition.

Abbreviation

DHA	Docosahexaenoic acid
EAAs	Essential Amino Acids
EFAs	Essential Fatty Acids
EPA	Eicosatetraenoic Acid
FAMEs	Fatty Acid Methyl Esters
GC-MS	Gas Chromatography-Mass Spectrometry
HPLC	High-Performance Liquid Chromatography
RSDs	Relative Standard Deviations
SIM	Selective Ion Monitoring

1. Introduction

Seahorses, belonging to the genus *Hippocampus*, represent a unique and fascinating group of fish species known for their distinctive horse-like head shape, prehensile tails, and unusual mode of reproduction. Found in both tropical and temperate waters around the world, seahorses typically inhabit shallow coastal areas, including seagrass beds, coral reefs, and mangroves [1]. In recent years, there has been a growing interest in the nutritional analysis of seahorses. Research has begun to explore the potential of seahorses as a source of nutrients, considering their use in potential dietary benefits [2–6]. This interest is driven by the need to understand the full spectrum of seahorse biology, including their nutritional value. They contain a diverse array of bioactive compounds, mainly including fatty acids and amino acids, which are considered the main constituents responsible for their dietary therapeutical properties. In this respect, a comprehensive analysis of fatty acids and amino acids are crucial for the potential application in health and dietary supplement.

Fatty acids and amino acids are fundamental components in nutritional studies due to their pivotal roles in human health and nutrition [7]. These organic compounds are essential in various physiological processes and are crucial for maintaining overall well-being. Fatty acids, particularly essential fatty acids (EFAs) like omega-3 and omega-6, are critical for numerous bodily functions. They are key components of cell membranes, influencing their fluidity and functionality. This is critical for normal cell operation, including the activities of membrane-bound enzymes and receptors. EFAs cannot be synthesized by the human body and thus must be obtained through diet, highlighting the importance of dietary sources rich in these fatty acids, especially in fish. Omega-3 fatty acids, such as eicosatetraenoic acid (EPA) and docosahexaenoic acid (DHA), are renowned for their anti-inflammatory properties. They play a crucial role in cardiovascular health, reducing the risk of heart diseases by lowering triglyceride levels, reducing blood pressure, and decreasing the likelihood of thrombosis [8]. DHA is also essential for brain health; it is a major structural component of the brain and is crucial for cognitive development and function [9]. Amino acids, the building blocks of proteins, are another class of compounds indispensable in nutrition. Proteins, made up of various amino acids, are involved in virtually every body function. From structural roles in building and repairing tissues, such as muscles and organs, to functional roles in enzymes, hormones, and antibodies, amino acids are integral to bodily health. There are 17 standard amino acids, of which nine are considered essential, meaning they cannot be synthesized by the body and must be obtained from the diet. Essential amino acids like lysine, tryptophan, and methionine are crucial for growth and development, immune function, and protein synthesis [10]. In addition to their structural and functional roles, some amino acids also act as precursors to neurotransmitters and other nitrogen-containing compounds. For instance, tryptophan is a precursor to serotonin, a neurotransmitter that plays a role in mood regulation.

The significance of fatty acids and amino acids in nutritional studies is also underlined by their dietary therapeutic potentials. Supplementing diets with these compounds has been studied for various health benefits, including improving mental health conditions, managing metabolic syndromes, and enhancing athletic performance [11–13]. In this way, understanding the content of these compounds in seahorses is fundamental for the development of dietary supplements. However, no reports have been reported regarding the quantification methods for fatty acids and amino acids in seahorses, nor have there been studies assessing the nutritional value of diverse seahorse species. Generally, current methods for analyzing fatty acids and amino acids primarily involve sophisticated chromatographic techniques. For fatty acids, Gas Chromatography coupled with Mass Spectrometry (GC-MS) is commonly employed. These methods involve the extraction and transesterification of fatty acids into fatty acid methyl esters for analysis. In addition, amino acid analysis typically utilizes High-Performance Liquid Chromatography (HPLC), where amino acids are often derivatized to enhance detection and quantification. Therefore, in the present study, we established a GC-MS for fatty acids and a HPLC for amino acids from

seahorses, respectively. More importantly, the nutritional value of eight different seahorses has also been investigated.

Previous studies on the nutritional and biochemical profiles of seahorses have provided valuable insights, but they have several limitations that our present study aims to address: (1) Limited Species Range: Most previous studies focused on a single seahorse species or a narrow range of species. For example, Cabral et al. examined the fatty acid profiles of cultured *Hippocampus hippocampus* [14], and Muhammadar et al. analyzed the amino acid profiles of seahorses from Simeulue waters [15]. (2) Comprehensive Profiling: Previous research often focused on either fatty acids or amino acids but not both. For instance, Abdullah Abbas et al. provided data on proximate composition and fatty acids but lacked amino acid profiling [16]. (3) Reproductive and Gender Differences: Previous studies often did not differentiate between genders or reproductive statuses [1,17,18]. By addressing these limitations, our study significantly broadens the understanding of seahorse biochemistry and nutrition, paving the way for more detailed and comprehensive future research.

2. Materials and methods

2.1. Seahorses and chemicals

Eight species of seahorses, including both genders of *Hippocampus trimaculatus*, *Hippocampus kelloggi*, *Hippocampus abdominalis*, and *Hippocampus erectus*, were obtained from various suppliers in Xiamen, China. The appearance of these seahorses is shown in Fig. 1. Chemicals used in the study, including solvents and reagents, were of analytical grade and purchased from reputable suppliers. The information of all chemicals used in the present study has been provided in Table 1.

2.2. GC-MS analysis for fatty acid measurement

2.2.1. Instrumentation and conditions

An Agilent HP-5MS column (30 m × 0.25 mm, 0.25 μm) was used for GC-MS analysis. Helium served as the carrier gas at a flow rate of 1.0 mL/min. The temperature program was as follows: (1) Initial temperature: 80 °C for 0.5 min; (2) Ramp to 165 °C at 20 °C/min, hold for 2 min; (3) Increase to 180 °C at 20 °C/min; (4) Final increase to 280 °C at 20 °C/min, hold for 10 min; (5) Inlet and detection temperatures were set at 250 °C. The electron ionization (EI) source was set at 70 eV with an ion source temperature of 230 °C. The mass scanning range was m/z 50–550 in SCAN mode with a solvent delay of 4 min. The injection volume was 1 μL.

2.2.2. Method validation

Linear Relationship Test: A mixed control solution of 34 fatty acid methyl esters (FAMEs) was analyzed. Selective Ion Monitoring (SIM) mode was used to select qualitative and quantitative ions for standard curve plotting (Fig. 2). Linear equations and other relevant parameters are provided in Table S1. Detection and quantification limits are detailed in Table 2.

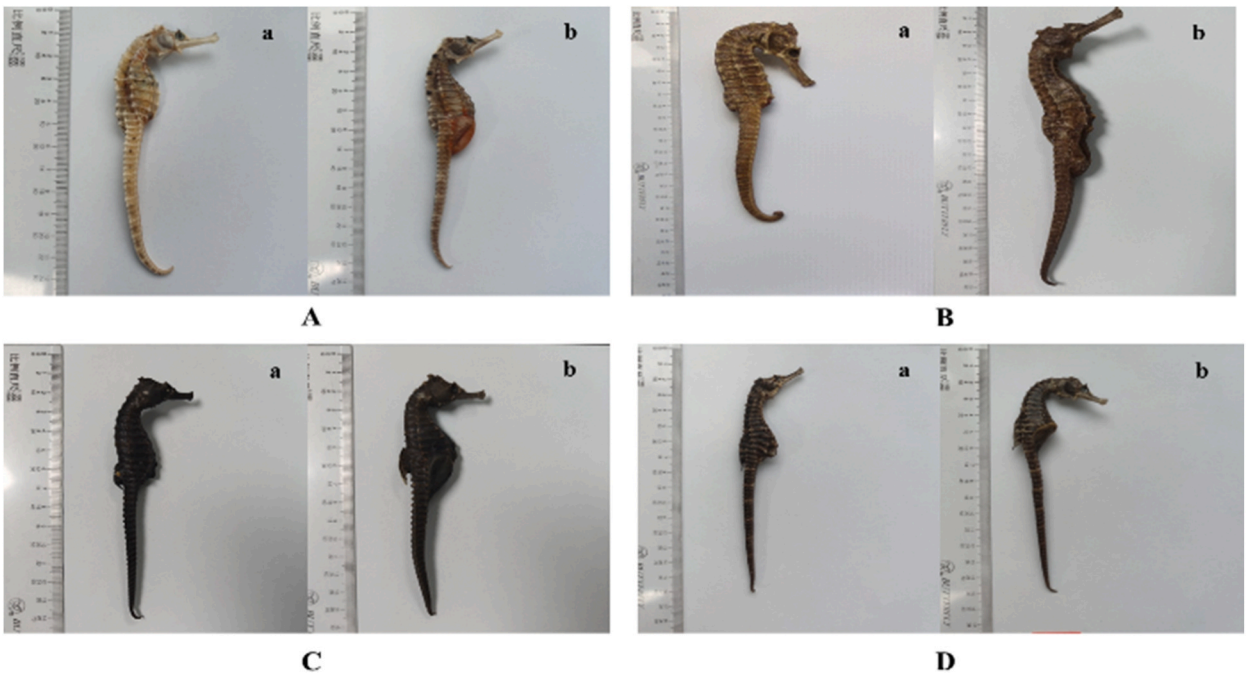

Fig. 1. Four kinds of hippocampal appearance traits. A : *Hippocampus trimaculatus*;B : *Hippocampus kelloggi*;C : *Hippocampus erectus*;D : *Hippocampus abdominalis*;a : Female , b : Male.

Table 1
The information of chemicals used in the present study.

No.	Name of Compound	CAS	Producer	Purity
1	Methyl caproat	106-70-7	Shanghai Anpel Experimental Technology Co., Ltd.	99.7 %
2	Methyl caprylate	111-11-5	Shanghai Anpel Experimental Technology Co., Ltd.	99.7 %
3	Methyl decanoate	110-42-9	Shanghai Anpel Experimental Technology Co., Ltd.	99 %
4	Methyl undecylate	1731-86-8	Shanghai Anpel Experimental Technology Co., Ltd.	99.7 %
5	Methyl dodecanoate (Methyl laurate)	111-82-0	Shanghai Anpel Experimental Technology Co., Ltd.	99 %
6	Tridecanoic acid methyl ester	1731-88-0	Shanghai Anpel Experimental Technology Co., Ltd.	99 %
7	*Cis*-9-tetradecenoic acid methyl ester (Myristic acid methyl ester)	56219-06-8	Shanghai Anpel Experimental Technology Co., Ltd.	99 %
8	Methyl myristate (Methyl myristate))	124-10-7	Shanghai Anpel Experimental Technology Co., Ltd.	99 %
9	10C-methyl pentadecenoate	90176-52-6	Shanghai Anpel Experimental Technology Co., Ltd.	99 %
10	Methyl pentadecanoate	7132-64-1	Shanghai Anpel Experimental Technology Co., Ltd.	99.8 %
11	*Cis*-9-hexadecenoic acid (Methyl palmitoleate)	1120-25-8	Shanghai Anpel Experimental Technology Co., Ltd.	99 %
12	Methyl hexadecanoate (Palmitic acid)	112-39-0	Shanghai Anpel Experimental Technology Co., Ltd.	99.5 %
13	Methyl *cis*-10-heptadecaenoate	75190-82-8	Shanghai Anpel Experimental Technology Co., Ltd.	99 %
14	Methyl-*n*-heptadecanoate	1731-92-6	Shanghai Anpel Experimental Technology Co., Ltd.	99 %
15	*Cis*-6,9,12-octadecadienoic acid (γ-linolenic acid methyl ester)	16326-32-2	Shanghai Anpel Experimental Technology Co., Ltd.	99 %
16	Methyl *cis*-9,12-octadecadienoate (Methyl linoleate)	112-63-0	Shanghai Anpel Experimental Technology Co., Ltd.	99.3 %
17	Methyl *cis*-9-octadecenoate (Methyl oleate)	112-62-9	Shanghai Anpel Experimental Technology Co., Ltd.	99.6 %
18	*Trans*-9,12-octadecadienoic acid methyl ester (Trans linoleic acid methyl ester)	2566-97-4	Shanghai Anpel Experimental Technology Co., Ltd.	99.7 %
19	*Trans*-9-octadecenoic acid methyl ester (Trans methyl oleate)	1937-62-8	Shanghai Anpel Experimental Technology Co., Ltd.	99.9 %
20	Methyl octadecanoate (Methyl stearate)	112-61-8	Shanghai Anpel Experimental Technology Co., Ltd.	99 %
21	All *cis*-5,8,11,14-tetracosenoic acid methyl ester (Arachidonic acid methyl ester, ARA)	2566-89-4	Shanghai Anpel Experimental Technology Co., Ltd.	99 %
22	Methyl 5,8,11,14,17-eicosapentaenoate (EPA)	2734-47-6	Shanghai Anpel Experimental Technology Co., Ltd.	99 %
23	Methyl *cis*-8,11,14-eicosatrienoate (Bis-homo-γ-linolenic acid)	21061-10-9	Shanghai Anpel Experimental Technology Co., Ltd.	99 %
24	Methyl *cis*-11,14-eicosadienoate	61012-46-2	Shanghai Anpel Experimental Technology Co., Ltd.	99 %
25	Methyl 11,14,17-cis-eicosatrienoate	55682-88-7	Shanghai Anpel Experimental Technology Co., Ltd.	99 %
26	Methyl eicosanoate (Methyl arachidate)	1120-28-1	Shanghai Anpel Experimental Technology Co., Ltd.	99 %
27	Methyl undecanoate	6064-90-0	Shanghai Anpel Experimental Technology Co., Ltd.	99 %
28	Methyl 4, 7, 10, 13, 16, 19-docosahexaenoate (DHA)	2566-90-7	Shanghai Anpel Experimental Technology Co., Ltd.	99 %
29	Methyl 13,16-cis-docosadienoate	61012-47-3	Shanghai Anpel Experimental Technology Co., Ltd.	99 %
30	Methyl *cis*-13-docosaenoate	1120-34-9	Shanghai Anpel Experimental Technology Co., Ltd.	99 %
31	Methyl docosanoate	929-77-1	Shanghai Anpel Experimental Technology Co., Ltd.	99 %
32	Methyl tricosanoate	2433-97-8	Shanghai Anpel Experimental Technology Co., Ltd.	99 %
33	Methyl *cis*-15-tetracosenoate	2733-88-2	Shanghai Anpel Experimental Technology Co., Ltd.	99 %
34	Methyl myristate	2442-49-1	Shanghai Anpel Experimental Technology Co., Ltd.	99 %

(*continued on next page*)

Table 1 (*continued*)

No.	Name of Compound	CAS	Producer	Purity
35	Lysine	56-87-1	Shanghai Anpel Experimental Technology Co., Ltd.	97.2 %
36	Glutamic acid	56-86-0	National Institutes for Food and Drug Control	100 %
37	Hydroxyproline	51-35-4	National Institutes for Food and Drug Control	99.9 %
38	Serine	56-45-1	National Institutes for Food and Drug Control	100 %
39	Glycine	56-40-6	National Institutes for Food and Drug Control	100 %
40	Histidine	71-00-1	National Institutes for Food and Drug Control	99.9 %
40	Arginine	74-79-3	National Institutes for Food and Drug Control	100 %
42	Threonine	72-19-5	National Institutes for Food and Drug Control	99.9 %
43	Alanine	56-41-7	National Institutes for Food and Drug Control	99.9 %
44	Proline	147-85-3	National Institutes for Food and Drug Control	100 %
45	Tyrosine	60-18-4	National Institutes for Food and Drug Control	99.9 %
46	Valine	72-18-4	National Institutes for Food and Drug Control	99.5 %
47	Methionine	63-68-3	National Institutes for Food and Drug Control	100 %
48	Isoleucine	73-32-5	National Institutes for Food and Drug Control	100 %
49	Leucine	61-90-5	National Institutes for Food and Drug Control	99.9 %
50	Phenylalanine	63-91-2	National Institutes for Food and Drug Control	100 %
51	Aspartic acid	56-84-8	National Institutes for Food and Drug Control	100 %

Precision Test: A 5-fold diluted control solution (2 μL) underwent six consecutive injections. Precision was assessed by the RSD of the peak areas of characteristic ions, with results in Table 2.

Repeatability Test: Six replicates of hippocampus powder test solution were prepared and analyzed in duplicate using both SCAN and SIM modes. The RSD of the peak area per unit mass was used to assess reproducibility (Table 2).

Stability Test: Seahorse samples were analyzed at intervals of 0, 3, 6, 9, 12, and 24 h. Stability was evaluated based on the RSD of peak areas of 12 FAMEs (Table 2).

Sample Recovery Test: Six seahorse samples were spiked with a 5-fold diluted mixed control solution and analyzed by GC-MS. Recovery rates were calculated by comparing the peak areas of characteristic ions to the standard curve (Table 3).

2.3. Fatty acid extraction methods

Two methods were compared for extracting total fatty acids.

(1) Trichloromethane-methanol method: 3.0 g of seahorse powder was placed in a stoppered conical flask. Then 30 mL of trichloromethane-methanol mixture (2:1, v/v) was added. The mixture was then subjected to ultrasonication using an ultrasonic apparatus (500 W, 45 KHz) for 30 min. Following ultrasonication, the mixture was filtered through filter paper. The extract was washed with 10 mL of 0.9 % sodium chloride solution, and the organic phase was collected. This was then evaporated to dryness in an 80 °C water bath and further dried at 50 °C until a constant weight was achieved.

(2) Pyrogallic gallic acid method: 3.0 g of seahorse powder was weighed into a stoppered conical flask. Then 0.1 g of pyrogallic acid, 2 mL of 95 % ethanol, and 4 mL of water were added, and the mixture was shaken well. Subsequently, 10 mL of 8.3 mol/L

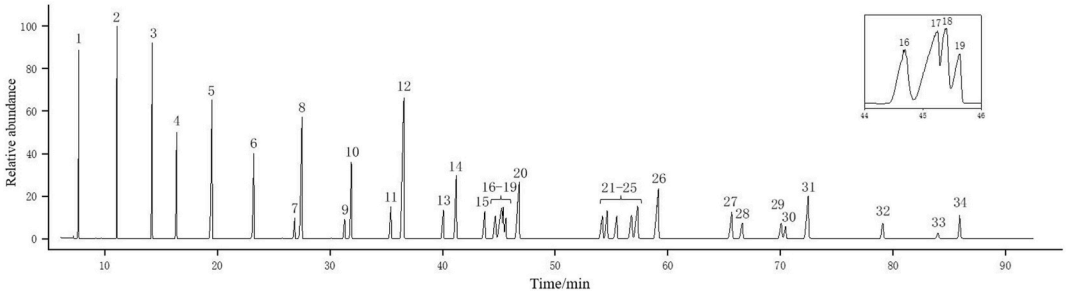

Fig. 2. Characteristic ion chromatograms of 34 fatty acid methyl ester mixed reference substances. 1.Methyl caproat; 2.Methyl caprylate; 3.Methyl decanoate; 4.Methyl undecylate; 5. Methyl laurate; 6.Tridecanoic acid methyl ester; 7.Myristic acid methyl ester; 8.Methyl *cis*-9-tetradecenoate; 9. Methyl myristate , Methyl myristate; 10.10C-methyl pentadecenoate; 11.Methyl-*n*-pentadecante、Methyl pentadecanoate; 12.Palmitoleic acid methyl ester/Hexadecenoic acid methyl ester (*cis*-9); 13.Methyl hexadecanoate/Methyl palmitate; 14.Methyl *cis*-10-heptadecaenoate; 15.Methyl heptadecanoate; 16.Methyl γ-linolenic acid/(Cis) −6,9,12-octadecadienoic acid; 17.Methyl *cis*-9,12-octadecadienoate; 18.Methyl *cis*-9-octadecenoate; 19.*Trans*-9,12-octadecadienoic acid methyl ester; 20.*Trans*-9-octadecenoic acid methyl ester; 21.Octadecanoic acid methyl ester; 22.Arachidonic acid methyl ester/*Cis*-5,8,11,14-tetracosenoic acid methyl ester; 23.Eicosapentaenoic acid methyl ester; 24.*Cis*-8,11,14-Eicosatrienoic acid methyl ester; 25.Methyl *cis*-11,14-eicosadienoate; 26.Methyl 11,14,17-cis-eicosatrienoate; 27.Methyl eicosanoate; 28.Methyl heneicosanoate; 29.All *cis*-4,7,10,13,16,19-docosahexaenoic acid methyl ester; 30.13,16-cis-docosadienoic acid methyl ester; 30.Methyl *cis*-13-docosaenoate; 31.Methyl docosacarbonate; 32.Methyl tricosanoate; 33.Methyl *cis*-15-tetracosenoate; 34.Methyl tetracosanoate.

Table 2
Methodological investigation of fatty acid methyl esters by GC-MS(n = 6).

No.	Name Of Fatty Acid Methyl Ester	Limit Of Detection (ng/mL)	Limit Of Quantitation (ng/mL)	Precision RSD(%)	Reproducibility RSD(%)	Stability RSD(%)
1	Methyl caproat	0.0561	0.1871	1.64	–	–
2	Methyl caprylate	0.0409	0.1364	1.53	–	–
3	Methyl decanoate	0.0574	0.1914	1.62	–	–
4	Methyl undecylate	0.0785	0.2617	1.53	–	–
5	Methyl dodecanoate (Methyl laurate)	0.0944	0.3146	1.63	–	–
6	Tridecanoic acid methyl ester	0.1041	0.3469	1.53	–	–
7	*Cis*-9-tetradecenoic acid methyl ester (Myristic acid methyl ester)	0.5000	1.6666	1.56	–	–
8	*Methyl myristate (Methyl myristate))	0.1104	0.3681	1.81	7.04	0.95
9	10C-methyl pentadecenoate	0.5391	1.7971	1.58	–	–
10	*Methyl pentadecanoate	0.1163	0.3876	1.82	8.37	1.05
11	**Cis*-9-hexadecenoic acid (Methyl palmitoleate)	0.0004	0.0013	1.58	6.71	1.32
12	*Methyl hexadecanoate (Palmitic acid)	0.1273	0.4243	1.94	6.77	1.41
13	Methyl *cis*-10-heptadecaenoate	0.0004	0.0014	1.69	–	–
14	*Methyl-*n*-heptadecanoate	0.1557	0.5191	1.91	6.72	1.57
15	*Cis*-6,9,12-octadecadienoic acid (γ-linolenic acid methyl ester)	0.0004	0.0013	1.84	–	–
16	*Methyl *cis*-9,12-octadecadienoate (Methyl linoleate)	0.0004	0.0013	1.87	6.67	2.82
17	*Methyl *cis*-9-octadecenoate (Methyl oleate)	0.0004	0.0014	2.16	–	–
18	*Trans*-9,12-octadecadienoic acid methyl ester (Trans linoleic acid methyl ester)	0.0004	0.0013	1.68	7.04	2.32
19	**Trans*-9-octadecenoic acid methyl ester (Trans methyl oleate)	0.0005	0.0018	2.25	6.55	2.40
20	*Methyl octadecanoate (Methyl stearate)	0.1913	0.6377	2.09	7.15	2.54
21	*All *cis*-5,8,11,14-tetracosenoic acid methyl ester (Arachidonic acid methyl ester, ARA)	0.0006	0.0019	1.96	8.67	3.27
22	*Methyl 5,8,11,14,17-eicosapentaenoate (EPA)	0.0005	0.0017	2.20	8.36	3.23
23	Methyl *cis*-8,11,14-eicosatrienoate (Bis-homo-γ-linolenic acid)	0.0006	0.0020	2.16	–	–
24	Methyl *cis*-11,14-eicosadienoate	0.0006	0.0021	2.07	–	–
25	Methyl 11,14,17-cis-eicosatrienoate	0.0012	0.0041	2.10	–	–
26	Methyl eicosanoate (Methyl arachidate)	0.3143	1.0478	2.53	–	–
27	Methyl undecanoate	0.4714	1.5714	2.53	–	–
28	*Methyl 4, 7, 10, 13, 16, 19-docosahexaenoate (DHA)	0.0009	0.0032	2.48	8.73	5.67
29	Methyl 13,16-cis-docosadienoate	0.0013	0.0043	2.77	–	–
30	Methyl *cis*-13-docosaenoate	0.0014	0.0046	2.17	–	–
31	Methyl docosanoate	0.6837	2.2788	3.26	–	–
32	Methyl tricosanoate	1.2161	4.0536	4.52	–	–
33	Methyl *cis*-15-tetracosenoate	0.0035	0.0115	5.12	–	–
34	Methyl myristate	1.8114	6.0379	5.25	–	–

hydrochloric acid was added, and the flask was placed in an 80 °C water bath for hydrolysis, shaking the mixture every 10 min for 40 min. After hydrolysis, 10 mL of 95 % ethanol was added and mixed thoroughly. The mixture was then transferred into a separatory funnel. The flask and stopper were washed with 50 mL of petroleum ether-ethyl ether (1:1) solution, which was also transferred to the separatory funnel. The mixture in the funnel was shaken for 5 min and allowed to stratify. The ether layer was collected into an evaporating dish. This extraction process was repeated three times. The separatory funnel was finally washed with petroleum ether - ethyl ether (1:1) solution into the evaporating dish. The total lipid extract was evaporated at 80 °C and dried at 50 °C until a constant weight was achieved.

The trichloromethane-methanol method yielded higher total lipids, making it the preferred method (Table S2).

2.4. Optimal methods for fatty acid methyl esterification

Four esterification methods were compared.

(1) Sulfuric acid methyl esterification method: Lipid extract reacted with sulfuric acid in methanol, followed by extraction with n-heptane and washing. In detail, 5.0 mg of total lipid extract was weighed and transferred to a reaction vessel. To this, 2 mL of 10 % sulfuric acid in methanol was added. The mixture was then heated in a 70 °C water bath for 20 min. After the reaction, the mixture was allowed to cool slightly before adding 10.0 mL of n-heptane, followed by vigorous shaking for 2 min. Subsequently, 10 mL of saturated sodium chloride solution was added. The mixture was allowed to stand until phase separation occurred. The

Table 3
Sample recovery test.

Reference Substance	Content Of Samples (μg)	Add Reference Volume (μg)	Measured (μg)	Recovery Rate (%)	Average Recoveries (%)	RSD (%)
Methyl myristate	0.0179	0.0836	0.0995	97.5403	99.79	3.91
	0.0197	0.0836	0.0998	95.7089		
	0.0165	0.0836	0.0998	99.5642		
	0.0152	0.0836	0.0962	96.9098		
	0.0228	0.0836	0.111	105.3982		
	0.0213	0.0836	0.1079	103.6133		
Methyl-*n*-pentadecante	0.0029	0.0412	0.0439	99.4506	100.89	3.36
	0.0034	0.0412	0.0432	96.5652		
	0.0023	0.0412	0.0439	101.0463		
	0.0018	0.0412	0.0425	98.6408		
	0.0044	0.0412	0.0479	105.4096		
	0.0037	0.0412	0.0466	104.2357		
Methyl *cis*-9-hexadecenoate	0.039	0.0431	0.0825	100.8778	109.12	7.31
	0.0412	0.0431	0.0862	104.2747		
	0.0356	0.0431	0.0832	110.3000		
	0.0332	0.0431	0.0774	102.3484		
	0.0453	0.0431	0.0972	120.0771		
	0.0421	0.0431	0.0925	116.8553		
Methyl palmitate	0.1836	0.1465	0.3183	91.9442	98.94	6.33
	0.1939	0.1465	0.3341	95.7232		
	0.1725	0.1465	0.3188	99.8885		
	0.1633	0.1465	0.3005	93.6001		
	0.2104	0.1465	0.3669	106.8769		
	0.1976	0.1465	0.3523	105.6206		
Methyl-*n*-heptadecanoate	0.0073	0.0397	0.0459	97.1241	99.54	4.02
	0.0079	0.0397	0.0456	94.9480		
	0.0064	0.0397	0.0459	99.3729		
	0.0058	0.0397	0.0444	97.1652		
	0.0091	0.0397	0.0509	105.2803		
	0.0081	0.0397	0.0492	103.3513		
Methyl *cis*-9,12-octadecadienoate	0.0194	0.076	0.0924	96.0967	99.2	4.54
	0.0209	0.076	0.0925	94.2158		
	0.0176	0.076	0.093	99.2080		
	0.0157	0.076	0.0894	96.9507		
	0.0238	0.076	0.1047	106.3926		
	0.0211	0.076	0.0989	102.3629		
Trans-9,12-octadecadienoic acid methyl ester	0.0427	0.0532	0.0924	93.4063	100.02	6.74
	0.0457	0.0532	0.0953	93.2464		
	0.0388	0.0532	0.0937	103.2196		
	0.036	0.0532	0.0869	95.5905		
	0.0506	0.0532	0.1074	106.7715		
	0.0462	0.0532	0.1036	107.8958		
Methyl pyruvate stearate	0.0746	0.0752	0.1437	91.8212	99.14	7.24
	0.0799	0.0752	0.1498	92.9744		
	0.0685	0.0752	0.1441	100.4969		
	0.0643	0.0752	0.1356	94.8188		
	0.0879	0.0752	0.1703	109.5991		
	0.0809	0.0752	0.1599	105.1244		
All *cis*-5,8,11,14-tetracosenoic acid methyl ester	0.0164	0.0365	0.0513	95.503	99.23	5.06
	0.0177	0.0365	0.052	93.9681		
	0.0146	0.0365	0.0508	99.1731		
	0.0136	0.0365	0.049	97.1763		
	0.0202	0.0365	0.0596	107.8405		
	0.0184	0.0365	0.0555	101.7115		
Methyl *cis*-5,8,11,14,17-eicosapentaenoate	0.0328	0.0348	0.0649	92.2408	98.73	7
	0.0352	0.0348	0.0675	92.8748		
	0.0298	0.0348	0.0644	99.5063		
	0.0278	0.0348	0.0609	94.9363		
	0.0392	0.0348	0.0776	110.2529		
	0.036	0.0348	0.0718	102.5918		
All *cis*-4,7,10,13,16,19-docosahexaenoic acid methyl ester	0.0736	0.0321	0.1021	88.9209	102.1	14.8
	0.0782	0.0321	0.1079	92.5001		
	0.0663	0.0321	0.0995	103.4636		
	0.0635	0.0321	0.0948	97.3108		
	0.0867	0.0321	0.1288	131.1374		
	0.0797	0.0321	0.1115	99.2487		

upper layer was then collected and mixed with 3g of anhydrous sodium sulfate, shaken for 1 min, and allowed to stand for 5 min. The clear upper layer was then transferred to a sample vial for analysis.

(2) Sodium hydroxide methyl esterification method: Lipid extract reacted with sodium hydroxide in methanol, followed by extraction with n-heptane and washing. In detail, 5.0 mg sample of total lipid extract was added to a reaction container. To this, 2 mL of 10 % sodium hydroxide in methanol was added, and the mixture was heated in a 70 °C water bath for 20 min. After cooling slightly, 10.0 mL of n-heptane was added, and the mixture was shaken for 2 min. This was followed by the addition of 10 mL of saturated sodium chloride solution. After standing to allow phase separation, the upper layer was collected, mixed with 3g of anhydrous sodium sulfate, shaken for 1 min, left to stand for 5 min, and then the upper layer was transferred to a sample vial.

(3) Sodium hydroxide - sulfuric acid methyl esterification method: Sequential reaction with sodium hydroxide and sulfuric acid in methanol, followed by extraction. In detail, 5.0 mg sample of total lipids was taken, and 2 mL of 10 % sodium hydroxide in methanol was added, followed by heating in a 70 °C water bath for 20 min. After evaporation, 2 mL of 10 % sulfuric acid in methanol was added, and the mixture was heated again in a 70 °C water bath for 20 min. Upon cooling, 10.0 mL of n-heptane was added, shaken for 2 min, followed by the addition of 10 mL of saturated sodium chloride solution. After phase separation, the upper layer was collected, mixed with 3g of anhydrous sodium sulfate, shaken for 1 min, allowed to stand for 5 min, and then the upper layer was transferred to a sample vial.

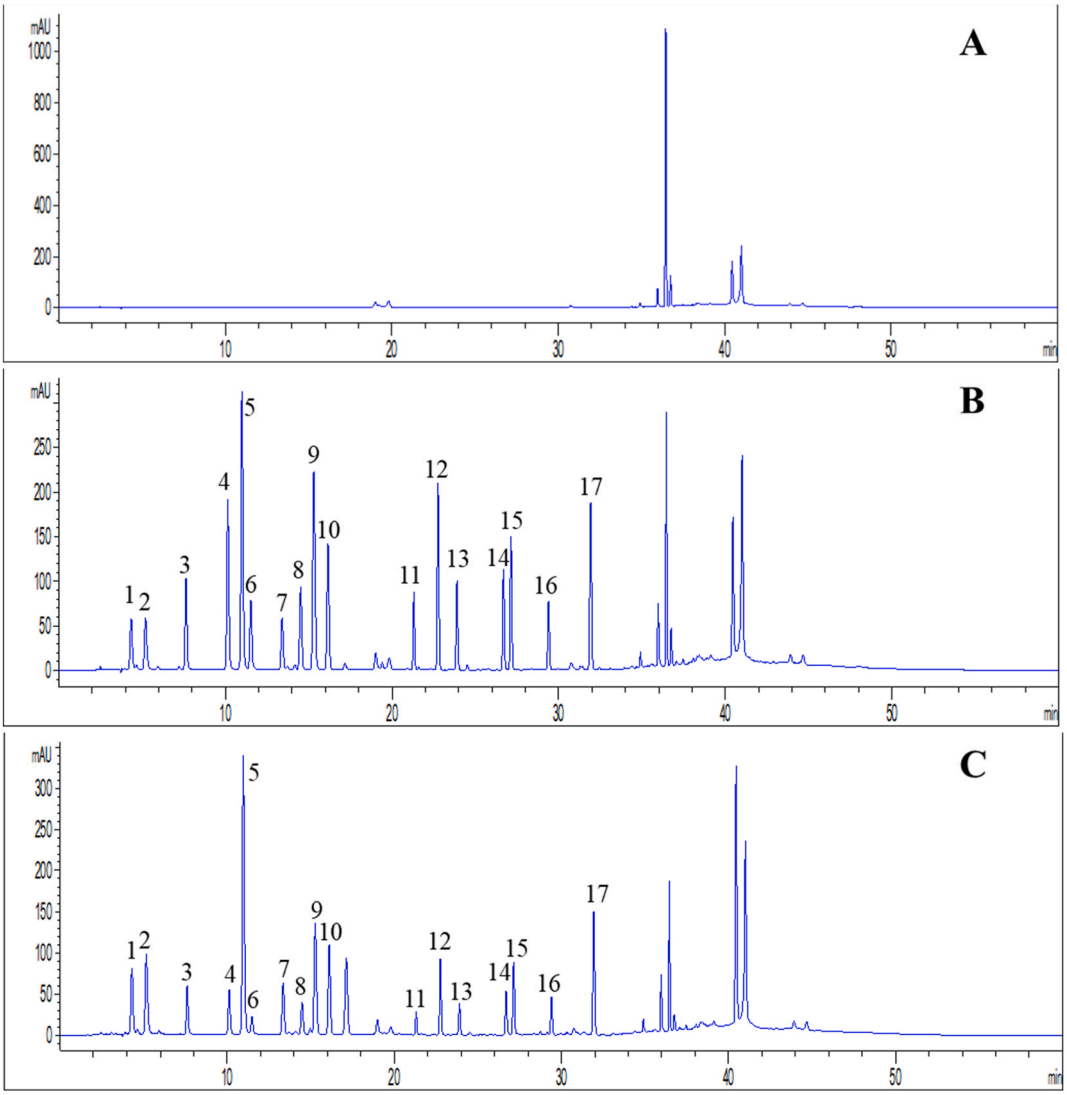

Fig. 3. High performance liquid chromatography of amino acids in different hippocampus. A Blank solution; B Mixed reference solution; C Hippocampal male test solution; 1: Asp 2: Glu 3: Hyp 4: Ser 5: Gly 6: His 7: Arg 8: Thr 9: Ala 10: Pro 11: Tyr 12: Val 13: Met 14: Ile 15: Leu 16: Phe 17: Lys.

(4) Sodium hydroxide - boron trifluoride methyl esterification method: Sequential reaction with sodium hydroxide and boron trifluoride in methanol, followed by extraction. In detail, 5.0 mg sample of total fat was weighed, and 4 mL of 2 % sodium hydroxide in methanol was added. The mixture was refluxed in an 80 °C water bath for 30 min. Subsequently, 5 mL of 15 % boron trifluoride in methanol was added from the top of the reflux condenser, and the mixture was refluxed for an additional 2 min. After cooling slightly, 10.0 mL of n-heptane was added, followed by shaking for 2 min, and then 10 mL of saturated sodium chloride solution was added. After phase separation, the upper layer was collected, mixed with 3g of anhydrous sodium sulfate, shaken for 1 min, left to stand for 5 min, and then transferred to a sample vial.

The sodium hydroxide-sulfuric acid method provided optimal esterification results.

2.5. HPLC for amino acid measurement

2.5.1. Instrumentation and conditions

HPLC analysis was performed with a mobile phase consisting of: Solution A: 0.1 mol/L sodium acetate (pH 6.5) and acetonitrile (93:7); Solution B: 80 % acetonitrile. A gradient elution method was used (Table S3). The flow rate was 1 mL/min, column temperature was 40 °C, detection wavelength was 254 nm, and injection volume was 2 μL.

2.5.2. Method validation

(1) System Suitability Test: Derivatization solutions of blank, mixed standards, and test samples were injected to evaluate separation efficiency (Fig. 3).
(2) Linear Relationship Test: Mixed control stock solutions were diluted, derivatized, and analyzed. Linear equations and detection limits are summarized in Table S4.
(3) Precision Test: A fourfold diluted amino acid mixture control was injected six times. RSD values are presented in Table 4.
(4) Repeatability Test: Six replicates of 0.04 g seahorse samples were analyzed. RSD values for amino acids are shown in Table 4.
(5) Stability Test: Seahorse samples were analyzed at intervals of 0, 2, 4, 6, 8, and 24 h post-derivatization. RSD values are shown in Table 4.
(6) Recovery Test: Seahorse amino acid hydrolysate was spiked with an amino acid control solution and analyzed post-derivatization. Recovery rates are presented in Table 5.

2.6. Preparation of seahorse amino acid hydrolysate

Seahorse samples (0.04 g) were hydrolyzed with 6 mol/L hydrochloric acid at 150 °C for 1 h. The hydrolysates were then filtered, evaporated, and dissolved in water for storage at 4 °C. Optimal hydrolysis time was determined based on the peak area of amino acids (Table S5).

2.7. Preparation of amino acid PITC derivatives

Amino acid mixed control solution and hippocampus amino acid hydrolysate were derivatized with triethylamine-acetonitrile and PITC-acetonitrile solutions, followed by extraction with n-hexane and filtration through a 0.45 μm membrane for HPLC analysis.

Table 4
HPLC methodological investigation of amino acids.

Amino Acid Name	Precision	Reproducibility	Stability
Aspartic acid	0.71	2.31	1.12
Glutamic acid	1.15	2.48	1.32
Hydroxyproline	0.45	2.73	1.42
Serine	0.38	1.90	0.65
Glycine	0.38	2.58	2.05
Histidine	0.55	2.25	1.53
Arginine	0.29	2.05	1.04
Threonine	1.23	1.93	2.86
Alanine	0.27	2.19	0.62
Proline	0.33	2.40	1.05
Tyrosine	0.26	1.59	0.62
Valine	0.41	2.20	0.66
Methionine	0.22	1.74	1.32
Isoleucine	0.23	2.68	1.57
Leucine	0.69	2.01	0.63
Phenylalanine	0.42	1.92	0.76
Lysine	0.57	1.78	2.73

Table 5
HPLC spiking recovery assay for amino acids in seahorses.

Amino Acid Name	Reproducibility						Average Recovery Rate	RSD	Recovery Rate Limit
	1	2	3	4	5	6			
Aspartic acid	79.94	77.74	80.74	79.39	79.78	78.43	79.34	1.37	92–105
Glutamic acid	101.62	95.21	98.69	96.67	97.24	96.05	97.58	2.36	92–105
Hydroxyproline	97.35	99.95	101.19	101.16	100.71	99.88	100.04	1.44	92–105
Serine	99.98	98.21	100.43	99.82	99.99	98.45	99.48	0.92	92–105
Glycine	99.20	97.79	100.94	100.23	100.98	100.02	99.86	1.21	92–105
Histidine	101.26	101.01	101.61	100.97	102.67	101.85	101.56	0.63	90–108
Arginine	104.43	99.53	101.83	100.09	99.15	97.88	100.49	2.31	92–105
Threonine	107.72	104.04	106.12	104.51	103.29	102.29	104.66	1.88	92–105
Alanine	102.07	100.04	102.15	100.72	100.77	99.57	100.89	1.04	92–105
Proline	102.16	99.25	102.82	101.39	101.74	100.78	101.36	1.23	92–105
Tyrosine	103.35	101.98	104.04	102.94	103.04	101.93	102.88	0.79	90–108
Valine	102.26	101.23	102.48	101.08	101.41	100.02	101.42	0.87	92–105
Methionine	106.62	107.33	107.97	106.58	107.52	106.78	107.13	0.53	92–105
Isoleucine	103.28	102.64	103.24	102.05	101.90	101.83	102.49	0.65	90–108
Leucine	99.90	96.64	99.26	96.16	97.19	96.66	97.63	1.59	92–105
Phenylalanine	101.38	101.50	102.26	101.59	101.59	100.70	101.50	0.49	92–105
Lysine	87.27	85.86	88.36	87.65	88.06	87.04	87.37	1.02	92–105

2.8. Data analysis

Data are presented as the mean ± SEM. Group comparisons were performed by a one-way ANOVA followed by LSD post hoc test in SPSS software. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Establishment of a GC-MS method for the determination of fatty acid content in seahorses

3.1.1. Precision and repeatability

The GC-MS method demonstrated high precision for detecting 34 fatty acid methyl esters, with relative standard deviations (RSDs) generally below 2.53 %. Exceptions were noted for methyl docosanoate, methyl eicosanoate, methyl *cis*-15-tetracosatetraenoate, and methyl eicosatetraenoate, which exhibited RSDs ranging from 3.26 % to 5.25 % (Table 2).

The repeatability test showed RSDs for 12 fatty acid methyl esters ranging between 6.55 % and 8.73 %, indicating satisfactory method repeatability (Table 2).

3.1.2. Stability and recovery

The stability test revealed that the RSDs for 12 fatty acid methyl esters were below 2.82 % within a 24-h period, with exceptions for methyl all-cis-5,8,11,14-eicosatetraenoate, methyl *cis*-5,8,11,14,17-eicosapentaenoate, and methyl all-cis-4,7,10,13,16,19-docosahexaenoate, which ranged from 3.23 % to 5.67 % (Table 2).

Sample recovery rates for major fatty acid methyl esters varied from 98.73 % to 109.12 %, meeting the required standards for content determination and indicating effective recovery (Table 3).

3.2. Fatty acid content and nutritional value in eight seahorse species

Significant variations in fatty acid content were observed among the eight seahorse species (Fig. 4). Female *Hippocampus erectus* showed higher levels of hexadecanoic acid and saturated fatty acids compared to both male and female Hippocampus abdominalis (Table 6). This difference may be attributed to dietary and metabolic factors, as discussed by previous studies [19,20].

The concentration of *cis*-9-octadecenoic acid was significantly greater in female *H. erectus* than in both male and female *H. abdominalis*, while other monounsaturated fatty acids were similar across these groups (Table 6). The higher levels of specific fatty acids in female *H. erectus* could be linked to reproductive needs and energy storage [21].

Male *H. abdominalis* exhibited notably lower concentrations of n-3 full cis 4,7,10,13,16,19-docosahexaenoic acid (DHA) compared to female H. trimaculatus (Table 6). DHA is essential for brain and eye development and overall health, suggesting that male *H. abdominalis* may have different dietary requirements or metabolic adaptations [22].

Total lipid yields varied from 3.2491 % to 12.3175 % of the dry product, with major fatty acids constituting 17.9717 %–74.6962 % of total lipids (Table 6). These findings indicate that seahorses are a valuable source of dietary lipids, particularly unsaturated fatty acids. The high $\sum$PUFAn-3/$\sum$PUFAn-6 ratios observed in seahorses exceed recommended values, highlighting their potential for human nutrition.

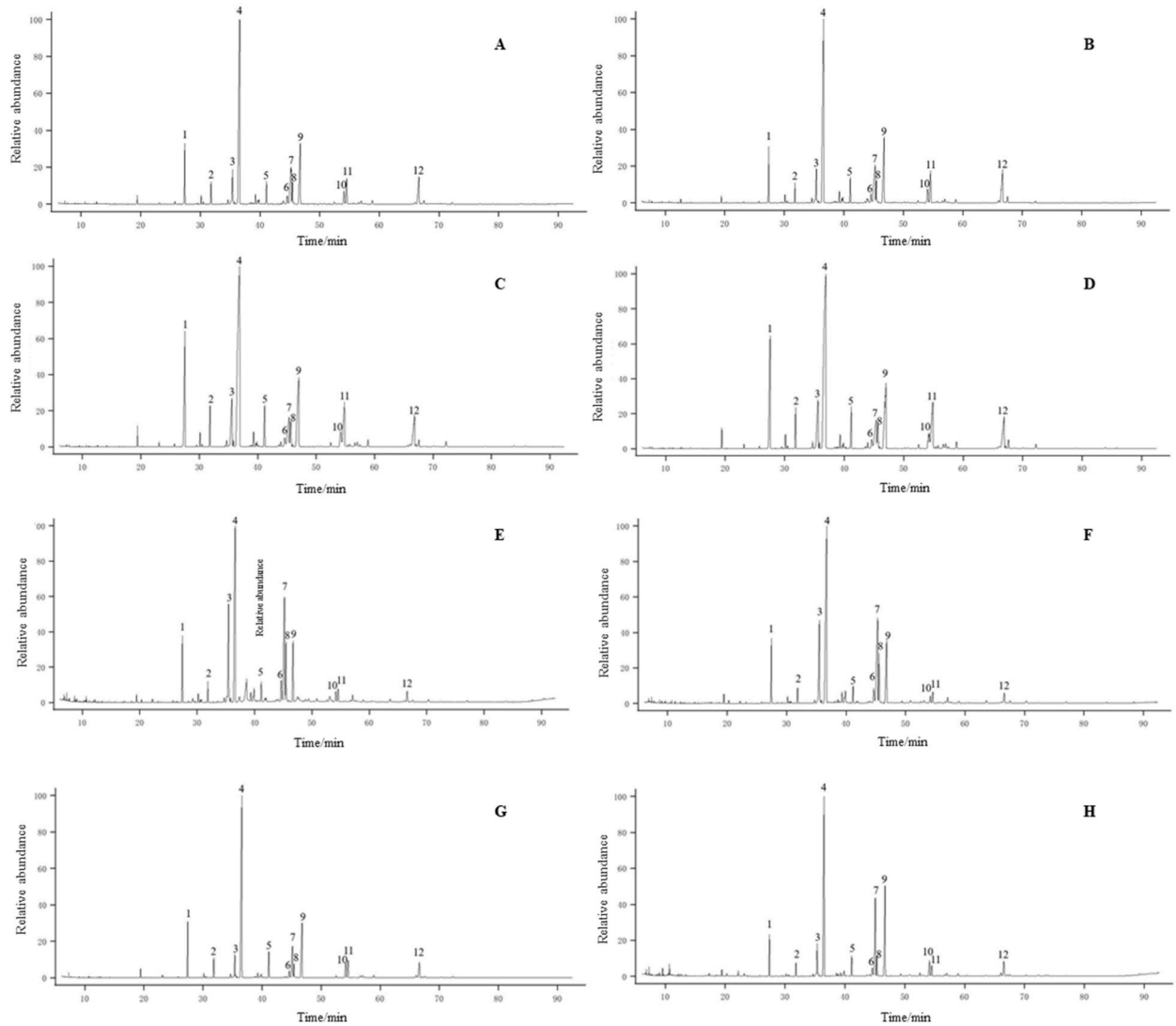

Fig. 4. GC-MS characteristic ion current plot of fatty acids in different hippocampus. A: Male *Hippocampus abdominalis;* B: Female *Hippocampus abdominalis;* C: Male *Hippocampus trimaculatus;* D: Female *Hippocampus trimaculatus;* E: Male *Hippocampus erectus;* F: Female G: Male *Hippocampus kelloggi;* H: Female *Hippocampus kelloggi.*

3.3. Establishment of an HPLC method for the determination of amino acid content in seahorses

3.3.1. Precision and reproducibility

The HPLC method provided a high degree of separation for 17 amino acid derivatives, with theoretical plate numbers exceeding 5000 and separation degrees all above 1.8. The linear relationship test showed correlation coefficients above 0.9980, indicating strong linearity (Table S4).

Instrumental precision was confirmed with RSDs for each peak area below 1.23 %, and the reproducibility of the sample preparation method was demonstrated by RSDs for the 17 amino acids being less than 2.73 % (Table 4).

3.3.2. Stability and recovery

The stability test showed RSDs of peak areas for 17 amino acid derivatives being less than 2.86 % within 24 h, indicating stability (Table 4). Recovery tests revealed that while aspartic acid and lysine showed lower average recoveries, the remaining 15 amino acids exhibited recovery rates ranging from 97.58 % to 104.66 %, further validating the robustness of the HPLC method (Table 5).

3.4. Amino acid content and nutritional value in eight seahorse species

Analysis of eight seahorse species revealed the presence of seven essential amino acids (EAAs), with the EAA/TAA ratio varying

Table 6
Total lipid yield of fatty acids in hippocampus, absolute content of saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids and fatty acid evaluation(%).

Chemicals	Hippocampus abdominalis male	Hippocampus abdominalis female	Hippocampus trimaculatus male	Hippocampus trimaculatus female	*Hippocampus erectus* male	*Hippocampus erectus* female	Hippocampus kelloggi male	Hippocampus kelloggi female
Palmitic acid	0.2170 ±0.0071	0.1907 ±0.0490	0.5280 ± 0.0628	0.5859 ± 0.0430	1.2961 ± 0.3377	2.2272 ± 0.2480[ab]	1.1062 ±0.0669	0.6422 ± 0.0195
Stearic acid	0.0958 ± 0.0029	0.0856 ± 0.0274	0.2236 ± 0.0320	0.2385 ±0.0171	0.2285 ± 0.0779	0.6251 ± 0.1357	0.5510 ±0.0227	0.3653 ± 0.0354
Myristic acid	0.0262 ± 0.0015	0.0156 ± 0.0067	0.1233 ± 0.0229	0.1611 ± 0.0147	0.1813 ± 0.0401	0.3913 ± 0.0594	0.1773 ±0.0081	0.0843 ± 0.0013
Margaric acid	0.0104 ± 0.0005	0.0084 ± 0.0036	0.0360 ±0.0058	0.0404 ± 0.0035	0.0460 ± 0.0029	0.1134 ± 0.0179	0.0964 ±0.0069	0.0495 ±0.0034
Pentadecylic acid	0.0063 ± 0.0002	0.0032 ± 0.0024	0.0242 ± 0.0038	0.0291 ± 0.0031	0.0402 ± 0.0107	0.0858 ± 0.0139	0.0593 ±0.0066	0.0264 ± 0.0010
Total saturated fatty acids	0.3556 ± 0.0113	0.3034 ± 0.0892	0.9352 ±0.1084	1.0550 ± 0.0812	1.7922 ± 0.4693	3.4428 ± 0.4749[ab]	1.9901 ±0.1112	1.1677 ± 0.0128
The proportion of saturated fatty acids in fatty acids	0.4494	0.4192	0.4592	0.4797	0.4615	0.5192	0.5222	0.5544
Cis-9-octadecenoic acid	0.1309 ± 0.0051	0.1178 ± 0.0348	0.1828 ± 0.0285	0.1814 ± 0.0214	0.6921 ± 0.1965	1.3085 ± 0.1717[ab]	0.6844 ±0.0586	0.3738 ± 0.0148
Cis-9 hexadecenoic acid	0.0462 ± 0.0051	0.0409 ± 0.0348	0.1899 ± 0.0285	0.2152 ± 0.0214	0.8015 ± 0.1965	1.2667 ± 0.1717	0.2875 ±0.0586	0.1403 ± 0.0148
Trans-9-octadecenoic acid	0.0580 ± 0.0027	0.0497 ± 0.0148	0.1316 ± 0.0201	0.1423 ± 0.0147	0.3664 ± 0.1188	–	0.2945 ±0.0203	0.1558 ± 0.0171
Cis-11-eicosenoic acid	0.0058 ±0.0006	–	–	–	–	–	–	–
Cis-10-heptadecaenoic acid	–	–	–	–	0.0479 ± 0.0085	0.1073 ± 0.0161	–	–
Total monounsaturated fatty acids	0.2409 ± 0.0096	0.2135 ± 0.0626	0.5142 ± 0.0739	0.5518 ± 0.0449	1.9078 ± 0.7725	2.6826 ± 0.3567	1.2664 ±0.1242	0.6699 ± 0.0302
The proportion of monounsaturated fatty acids in fatty acids	30.4239	29.4774	24.7075	25.0977	48.1718	40.4623	33.2400	31.8036
n-3 *cis*-4,7,10,13,16,19-docosahexaenoic acid	0.0926 ± 0.0153[d]	0.1035 ± 0.0305	0.1916 ± 0.0314	0.2100 ± 0.0538	–	–	–	–
n-3 *cis*-5,8,11,14,17-eicosapentaenoic acid	0.0411 ± 0.0029[cd]	0.0440 ± 0.0124	0.1479 ± 0.0222	0.2013 ± 0.0179	0.0825 ± 0.0643	0.1566 ± 0.0181	0.0818 ±0.0991	0.0570 ± 0.0018
n-6 *cis*-9,12-octadeca-dienoic acid	0.0260 ± 0.0016	0.0219 ± 0.0075	0.2017 ± 0.3026	0.0620 ± 0.0055	–	–	–	–
n-6 *cis*-5,8,11,14-eicosatetraenoic acid	0.0218 ± 0.0013	0.0181 ± 0.0063	0.0559 ± 0.0086	0.0638 ± 0.0053	0.0602 ± 0.0421	0.1004 ± 0.0139	0.1636 ±0.0122	0.0803 ± 0.0013
n-3 *cis*-7,10,13,16,19-docosapentaenoic acid	–	0.0199 ± 0.0067	–	0.0548 ± 0.0045	–	–	–	–
Total polyunsaturated fatty acids	0.1952 ±0.0165[cd]	0.2074 ±0.0634[cd]	0.6318 ± 0.3494	0.5919 ± 0.0484	–	–	–	–
Polyunsaturated fatty acids in fatty acids	24.6575	28.6305	30.3563	26.9211	0.0658	0.0761	0.1452	0.1276

(*continued on next page*)

Table 6 (*continued*)

Chemicals	Hippocampus abdominalis male	Hippocampus abdominalis female	Hippocampus trimaculatus male	Hippocampus trimaculatus female	*Hippocampus erectus* male	*Hippocampus erectus* female	Hippocampus kelloggi male	Hippocampus kelloggi female
The total lipid yield was calculated according to the dried product	4.0329	5.0923	6.7083	8.2323	8.7758	12.3175	7.5322	3.2491
Absolute content of total fatty acids	0.7917 ± 0.0338^{cdefgh}	0.7243 ±0.02152^{cdefgh}	2.0812 ± 0.4845	2.1988 ± 0.1439	3.9605 ± 1.4397	6.6299 ± 0.8944	3.8098 ±0.1645	2.1065 ± 0.0489
The proportion of fatty acids in total lipids	22.2082	17.9717	36.492	31.2677	52.1298	37.3852	59.84	74.6962
Total unsaturated fatty acids	0.4361 ± 0.0249^{cdefgh}	0.4209 ± 0.1260^{cdefgh}	1.1460 ± 0.4042	1.1438 ± 0.0811	2.1683 ±0.9703	3.1870 ± 0.4195	1.8197 ±0.0532	0.9388 ± 0.0362
The proportion of unsaturated fatty acids in fatty acids	55.0814	58.1080	55.0638	52.0187	53.8532	48.0819	47.7774	44.5593
Total n-3 polyunsaturated fatty acids	0.1474 ± 0.0155	0.1674 ± 0.0497	0.3742 ± 0.0595	0.4661 ± 0.0487	0.2003 ± 0.1557	0.4041 ± 0.0488	0.3897 ±0.0832	0.1886 ± 0.0047
Total n-6 polyunsaturated fatty acids	0.0478 ± 0.0030	0.0400 ± 0.0137	0.2576 ± 0.3081	0.1258 ± 0.0108	0.0602 ± 0.0421	0.1004 ± 0.0139	0.1636 ±0.0122	0.0803 ± 0.0013
PUFAn-3/PUFAn-6	3.0864	4.2374	2.7216	3.7269	3.2073	4.031	2.4082	2.3491

Note：a, b, c, d, e, f, g, h represent male of Hippocampus abdominalis, female of Hippocampus abdominalis, male of Hippocampus trimaculatus, female of Hippocampus trimaculatus, male of *Hippocampus erectus*, female of *Hippocampus erectus*, male of Hippocampus kelloggi, and female of Hippocampus kelloggi, respectively. Fatty acids with "- "were not detected fatty acids or fatty acids with characteristic ion peak area below 1 % of the total peak area. The content of *Cis*-11-eicosenoic acid was determined by the standard curve of Methyl 11,14,17-cis-eicosatrienoate. The content of *cis*-7,10,13,16,19-docosapentaenoic acid was determined by the standard curve of 13.16-cis-docosadienoic acid methyl ester. There are three kinds of polyunsaturated fatty acids PUFAn-3: *cis*-5,8,11,14,17-eicosapentaenoic acid, *cis*-4,7,10,13,16,19-docosahexaenoic acid,*cis*-7,10,13,16,19-docosapentaenoic acid; there are two kinds of polyunsaturated fatty acids PUFAn-6: *cis*-9,12-octadecadienoic acid and *cis*-5,8,11,14-eicostetraenoic acid. SPSS 23.0 statistical software was used for statistical analysis of the data. Analysis of variance was used among multiple groups. The upper right corner was marked as that the data were statistically different from the same row of the labeled samples ($P < 0.05$).

Table 7
Hippocampal amino acid content and amino acid characteristic index value.

Items	classification of amino acids	Hippocampus abdominalis male	Hippocampus abdominalis female	Hippocampus trimaculatus male	Hippocampus trimaculatus female	Hippocampus kelloggi male	Hippocampus kelloggi female	*Hippocampus erectus* male	*Hippocampus erectus* female
Aspartic acid(Asp) (g/100g)	bcg	3.14	2.55	2.37	1.97	2.08	2.37	2.61	2.36
Glutamic acid(Glu) (g/100g)	bcg	5.31	4.72	4.48	3.69	4.01	4.63	5.06	4.38
Hydroxyproline(Hyp) (g/100g)	b	1.92	1.78	2.58	2.12	2.18	2.53	2.59	2.02
Serine(Ser) (g/100g)	b	2.18	2.05	1.84	1.57	1.55	1.62	1.98	1.85
Glycine(Gly) (g/100g)	bcg	7.35	7.41	9.45	8.15	8.14	8.77	9.15	7.24
Histidine(His) (g/100g)	bf	0.83	0.76	0.55	0.25	0.50	0.66	0.67	0.61
Arginine(Arg) (g/100g)	bg	3.27	3.10	2.91	2.41	2.64	3.23	3.33	2.86
Threonine(Thr) (g/100g)	a	1.81	1.56	1.19	0.94	1.11	1.45	1.50	1.35
Alanine(Ala) (g/100g)	bc	3.70	3.53	4.25	3.66	3.82	4.08	4.37	3.73
Proline(Pro) (g/100g)	b	3.89	3.61	4.61	3.59	4.04	4.62	4.94	4.02
Tyrosine(Tyr) (g/100g)	bef	0.91	0.80	0.56	0.59	0.50	0.68	0.70	0.71
Valine(Val) (g/100g)	ad	1.59	1.45	0.98	0.76	0.90	1.46	1.22	1.08
Methionine(Met) (g/100g)	afg	1.22	1.14	1.11	0.97	1.00	1.10	1.24	1.10
Isoleucine(Ile) (g/100g)	adg	0.58	0.54	0.32	0.27	0.31	0.50	0.40	0.39
Leucine(Leu) (g/100g)	adg	4.36	3.90	2.93	2.43	2.74	3.27	3.47	3.36
Phenylalanine(Phe) (g/100g)	aeg	1.40	1.23	0.94	0.74	0.85	1.12	1.16	1.04
Lysine(Lys) (g/100g)	ag	2.95	2.54	2.09	1.66	1.70	2.33	2.30	2.07
Cystine(Cys) (g/100g)	af	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Total contents of amino acids(TAA) (g/100g)	/	46.41	42.67	43.17	35.77	38.05	44.42	46.68	40.18
Essential amino acid EAA(g/100g)	/	13.91	12.35	9.57	7.77	8.59	11.22	11.29	10.40
Nonessential amino acid NEAA(g/100g)	/	32.50	30.32	33.59	28.00	29.45	33.20	35.39	29.78
Essential amino acids for children CEAA (g/100g)	/	18.01	16.22	13.03	10.42	11.73	15.11	15.29	13.87
Delicious amino acids AA(g/100g)	/	19.50	18.21	20.55	17.47	18.05	19.85	21.18	17.71
Branched chain amino acid BCAA(g/100g)	/	6.54	5.89	4.24	3.46	3.94	5.22	5.09	4.84
Aromatic amino acid AAA(g/100g)	/	2.30	2.03	1.50	1.33	1.35	1.81	1.86	1.76
Antioxidant amino acids ATAA(g/100g)	/	2.96	2.69	2.22	1.82	1.99	2.44	2.60	2.42

(*continued on next page*)

Table 7 (*continued*)

Items	classification of amino acids	Hippocampus abdominalis male	Hippocampus abdominalis female	Hippocampus trimaculatus male	Hippocampus trimaculatus female	Hippocampus kelloggi male	Hippocampus kelloggi female	*Hippocampus erectus* male	*Hippocampus erectus* female
Medicinal amino acids MAA(g/100g)	/	29.58	27.14	26.60	22.28	23.46	27.32	28.72	24.81
EAA/TAA(%)	/	29.96	28.95	22.18	21.71	22.59	25.26	24.18	25.89
NEAA/TAA(%)	/	70.04	71.05	77.82	78.29	77.41	74.74	75.82	74.11
CEAA/TAA(%)	/	38.81	38.00	30.18	29.14	30.82	34.02	32.75	34.52
FAA/TAA(%)	/	42.01	42.68	47.60	48.85	47.44	44.70	45.39	44.07
BCAA/TAA(%)	/	14.09	13.80	9.81	9.66	10.36	11.76	10.90	12.04
ATAA/TAA(%)	/	6.37	6.31	5.15	5.08	5.24	5.50	5.58	6.01
MAA/TAA(%)	/	63.74	63.59	61.63	62.29	61.66	61.50	61.52	61.74
EAA/NEAA(%)	/	42.78	40.75	28.50	27.73	29.18	33.79	31.89	34.93
BCAA/AAA specific value	/	2.84	2.90	2.82	2.59	2.92	2.89	2.74	2.75

Note：a essential amino acid (EAA); b Non-essential amino acids (NEAA); c umami amino acids (FAA); d branched chain amino acids (BCAA); e aromatic amino acids (AAA); f antioxidant amino acids (ATAA); g medicinal amino acids (MAA).

from 29.96 % in male puffed-bellied seahorses to 21.71 % in female three-spotted seahorses (Table 7). This variation can be attributed to dietary sources and environmental factors.

The CEAA/TAA ratios, which include histidine and arginine, ranged from 38.81 to 29.14, suggesting potential nutritional benefits for children (Hou and Wu, 2018). The presence of nine amino acids with medicinal properties in all eight species further emphasizes their health benefits [23].

The BCAA/TAA ratios ranged from 14.09 to 9.66, with male puffed-bellied seahorses having the highest ratio. BCAAs are known for promoting protein synthesis and enhancing immunity. The BCAA/AAA ratio, significant for liver disease treatment, ranged from 2.90 to 2.59 (Table 7).

4. Discussion

In this study, the application of GC-MS and HPLC provided detailed insights into the fatty acid and amino acid profiles of seahorses. The GC-MS analysis demonstrated high precision in detecting fatty acids, with RSDs for the peak areas of 34 fatty acid methyl esters generally below 2.53 %. Furthermore, the repeatability of the method was affirmed by RSDs for 12 fatty acid methyl esters, which ranged between 6.55 % and 8.73 %. In terms of stability, these fatty acids maintained RSDs under 2.82 % within a 24-h period, showcasing the method's reliability. The recovery rates for these fatty acids were also noteworthy, varying from 98.73 % to 109.12 %, which further underscores the effectiveness of the GC-MS method. On the other hand, HPLC analysis revealed a high degree of separation and strong linear correlations for the 17 studied amino acid derivatives. Theoretical plate numbers exceeded 5000, with separation degrees all above 1.8, reflecting the column's high efficacy. Instrumental precision was confirmed with RSDs for each peak area below 1.23 %. Additionally, the reproducibility of sample preparation was demonstrated by RSDs for the 17 amino acids being less than 2.73 %, while the stability tests showed RSDs less than 2.86 % over 24 h. Recovery tests for the amino acids indicated recovery rates ranging from 97.58 % to 104.66 %, further validating the robustness of the HPLC method for amino acid analysis in seahorse samples. Taken together, these results from GC-MS and HPLC analyses highlight the precision, repeatability, stability, and effective recovery rates of the methods, making them reliable techniques for analyzing biochemical components in seahorse samples.

The fatty acid content and composition in seahorses are of significant interest, particularly from a nutritional and ecological perspective. Fatty acids, especially PUFAs, are essential components in marine organisms, contributing to various biological functions, including membrane fluidity, energy storage, and signaling processes [24]. Our findings on the fatty acid profiles across different seahorse species offer valuable insights into their dietary habits, metabolic processes, and potential nutritional value. A noteworthy observation is the higher concentration of hexadecanoic acid and saturated fatty acids in female *H. erectus* compared to *H. abdominalis*. This difference could be attributed to several factors, including dietary variations, habitat preferences, and physiological differences between species [19,20]. Seahorses primarily feed on small crustaceans and plankton, and the fatty acid composition of their diet is directly reflected in their own fatty acid profiles [25]. Therefore, the variation in fatty acid content could indicate differences in the available prey and feeding behaviors among these species. Additionally, variations in habitat, such as water temperature, salinity, and the presence of pollutants, can influence the fatty acid composition of seahorses [26]. The elevated levels of saturated fatty acids, including hexadecanoic acid, in female *H. erectus* might also be linked to reproductive factors. In seahorses, females transfer eggs to the male's brood pouch, where fertilization and embryonic development occur [27]. The higher content of certain fatty acids in females could be related to their role in egg production, providing essential nutrients for embryonic development [21]. Conversely, the lower concentration of certain fatty acids, such as DHA, in specific seahorse species like male *H. abdominalis*, raises interesting biological questions. DHA is a crucial omega-3 fatty acid, known for its role in brain and eye development, as well as overall health [22]. The reduced levels of DHA could be linked to the dietary composition or the efficiency of these seahorses in assimilating and metabolizing omega-3 fatty acids. It might also reflect adaptive mechanisms to specific environmental conditions, as fatty acid metabolism can vary in response to external factors like temperature and food availability. The implications of these variations are also significant for understanding seahorse biology and ecology. For instance, the fatty acid composition can influence the quality of the brood pouch environment in males and may affect reproductive success and offspring survival [28]. From a conservation standpoint, understanding these nutritional aspects is important for effective species management and habitat preservation.

The fatty acid profiles of seahorses, especially the high levels of unsaturated fatty acids and their ratios ($\sum$PUFAn-3/$\sum$PUFAn-6), have noteworthy nutritional implications. Unsaturated fatty acids, including omega-3 and omega-6 fatty acids, are essential for human health, playing crucial roles in brain function, inflammation regulation, and heart health [29]. The $\sum$PUFAn-3/$\sum$PUFAn-6 ratio is particularly significant as it reflects the balance between these two types of fatty acids, which is crucial for maintaining optimal health [30]. A diet with a balanced ratio of omega-3 to omega-6 fatty acids is associated with reduced risk of chronic diseases such as heart disease, cancer, and inflammatory disorders [31]. The findings from our study showed that the $\sum$PUFAn-3/$\sum$PUFAn-6 ratios in seahorses exceeded these recommended values, ranging from 2.3491 to 4.2374. This indicates a predominance of omega-3 fatty acids in seahorses, which is a desirable dietary feature, considering the typical Western diet is often disproportionately high in omega-6 fatty acids [32]. The high omega-3 content in seahorses, particularly the presence of DHA and EPA, is of significant interest for human nutrition. DHA and EPA have been extensively studied for their beneficial effects on heart health, brain development, and as anti-inflammatory agents. The elevated levels of these fatty acids in seahorses suggest their potential as a supplement, especially in diets deficient in omega-3 fatty acids.

The EAA content in seahorses and its variability among species presents a fascinating area of study with significant nutritional implications [33]. EAAs are amino acids that cannot be synthesized by the body and must be obtained through diet. They are crucial for various bodily functions, including tissue growth, energy production, and immune system support. Our analysis revealed that all eight seahorse species studied contain seven essential amino acids. However, the EAA content varied among species, as indicated by

EAA/TAA and EAA/NEAA. These variations can be attributed to differences in diet, habitat, and physiological adaptations of each species. The dietary source of seahorses, primarily small crustaceans and plankton, likely influences their amino acid profile. Additionally, environmental factors such as water temperature and salinity may affect the availability and type of food sources, thus impacting the EAA content. The potential nutritional benefits of these EAAs, particularly for specific demographics like children, are noteworthy. Children require adequate EAAs for growth, development, and overall health. The CEAA/TAA ratios, which include histidine and arginine in addition to the seven EAAs, were found to range from 38.81 to 29.14 among the seahorse species. These ratios are crucial as they reflect the adequacy of EAAs in the diet. While the ratios varied among species, they suggest that seahorses could potentially be a supplementary source of EAAs for children, especially in diets where essential nutrient intake might be lacking. Moreover, the presence of nine amino acids with known medicinal properties (Asp, Gly, Glu, Met, Leu, Tyr, Phe, Lys, and Arg) in all studied seahorse species is of particular interest. These amino acids are known for their various health benefits. For instance, Arginine is important for wound healing and immune function [23], Methionine acts as a powerful antioxidant [34], and Leucine plays a critical role in muscle protein synthesis [35]. The high ratio of MAA/TAA further emphasizes the potential health benefits of seahorses. These findings suggest that seahorses could be a valuable source of both essential and medicinal amino acids, contributing to dietary diversity and potentially offering health benefits.

Our study provides a comprehensive biochemical analysis and nutritional evaluation of eight seahorse species (Hippocampus spp.), including both genders of *Hippocampus trimaculatus*, *Hippocampus kelloggi*, *Hippocampus abdominalis*, and *Hippocampus erectus*. This work builds on existing research by offering a broader scope and depth of analysis. Previous studies, such as the analysis of fatty acid profiles in *Hippocampus hippocampus* trunk muscles, focused on single species and specific body parts [14]. In contrast, our research encompasses a diverse range of species and includes both fatty acid and amino acid profiles across the entire organism. The amino acid profile study on seahorses from Simeulue waters also focused on a single species and specific amino acids [15]. Our study, however, provides a holistic nutritional evaluation by analyzing both amino acids and fatty acids in multiple species, highlighting interspecies and gender-specific variations. The study on Hippocampus comes compared cultured versus wild specimens [36], whereas our research does not limit the comparison to cultured and wild but rather focuses on the inherent biochemical differences among species. Additionally, while the investigation into marine amphipods as an alternative feed for *Hippocampus erectus* examined dietary effects on a single species [18], our study's broader species range offers foundational data for future studies on diet and nutrition across different seahorse species. Research on the proximate composition and fatty acids of seahorses from Simeulue provided valuable regional data but did not include amino acid profiling [16]. Our study expands on this by offering both proximate and biochemical analyses, providing a more detailed nutritional assessment. Studies that examined the effects of different temperatures and diets on juvenile seahorses and the impact of lipid-enriched diets on growth performance provided insights into environmental and dietary influences on single species [1,17]. Our research, however, provides baseline biochemical composition data across multiple species, crucial for understanding nutritional value independent of external variables. Anyway, while previous studies have provided valuable insights into specific aspects of seahorse biochemistry and nutrition, our research significantly broadens the scope by incorporating a diverse range of species and comprehensive biochemical profiling.

5. Conclusion

In conclusion, this study represents an advancement in our understanding of the biochemical composition of seahorses, offering a comprehensive analysis of both fatty acid and amino acid profiles across multiple seahorse species. The application of GC-MS and HPLC techniques has not only provided a detailed nutritional profile of these fascinating marine creatures but also uncovered unique aspects of their biochemical makeup. Key findings include the high levels of unsaturated fatty acids, particularly omega-3 fatty acids, with notably high $\sum$PUFAn-3/$\sum$PUFAn-6 ratios that surpass common values in many marine organisms. This unique fatty acid composition indicates the potential of seahorse as a rich source of essential nutrients. Additionally, the study has illuminated the presence of essential amino acids, including those with medicinal properties, further enhancing the nutritional and therapeutic value of seahorses.

Ethics approval

No ethical approval was required for this study as this research didn't involve any human or experimental animal subjects.

Data available statement

Data will be made available on request.

CRediT authorship contribution statement

Bi-Yun Feng: Writing – original draft, Investigation, Formal analysis. **Hui Zhang:** Investigation, Formal analysis. **Dong-Yuan Zhang:** Investigation, Formal analysis. **You-Hua Luo:** Formal analysis. **Hui Yang:** Formal analysis. **Jing Lin:** Investigation, Formal analysis. **Ling-Yan Li:** Formal analysis. **Xian-Zhu Qiu:** Formal analysis. **Feng-Yan Qiu:** Investigation. **Li-Shan Ye:** Formal analysis, Conceptualization. **Li-Tao Yi:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Guang-Hui Xu:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

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