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# GC-MS Analysis of Amino Acids in Food Chain of Key Species Anchovy (*Engraulis japonicus*) of the Yellow Sea and East China Sea Food Web

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*Calanus sinicus* and *Engraulis japonicus* were used in the experiment to understand the transfer process of amino acids in food chain. Through 76 days' experiment, a quantitative analysis of 14 kinds of amino acid content was carried in collected samples. The results showed that, *Calanus sinicus* played a connecting role in the food chain, it not only transferred plant protein into animal protein, greatly improved lysine content, but also increased total amino acid content of chlorella from 6.10 to 24.16 %. As the main economic fish in the food chain, *Engraulis japonicus* further increased amino acid content to 27.06 %. In terms of the relationship between *Calanus sinicus*'s amino acid composition and *Chlorella* ( $r = 0.606$, $p < 0.05$ ), the correlation of 7 essential amino acids was relatively weak, while the correlation of 7 non-essential amino acids was obviously stronger. When anchovy's bait was changed from synthetic bait to *Calanus sinicus*, its amino acid content changed to some degree, before the change, it was related to synthetic bait, after the change, it was more closely related to *Calanus sinicus*. Amino acid content of anchovy feces was mainly decided by physiological processes of metabolism.

**Keywords: Amino Acid, Food Chain, Yellow Sea and East China Sea Ecological System.**

## INTRODUCTION

The Yellow sea and East China sea ecological system is one of the most important marine ecosystems in China, it has rich biological diversity and complex food web structure. The East China sea has 727 species of fish, 200 kinds of fishery objects. The Yellow sea has 289 kinds of fish and 100 kinds of fishery objects[1]. In recent years, China's marine fishery production is constantly increasing, but there are obvious problems, such as large decrease of some high quality traditional fish resources and increase of low value fish. People have little understanding of function and mechanism of coastal ecosystem, quantitative food relationship, the relationship between energy flow and conversion and nutritional quality are urgent problems to be solved. In the Yellow sea and East China sea food chain, *Engraulis japonicus* belongs to primary carnivorous anima, it is the prey for 40 species of predators. Japanese scholars have studied nutrition change of *Engraulis japonicus* in early stage, seasonal variation of its food organism, predation specifications and diet composition and a series of other problems[2-4]. In the national 973 project" Dynamics and Sustainable Use of Biological Resources in the Yellow Sea and East China Sea Ecosystem, phytoplankton dynamics-*phytoplankton dynamics* and *Calanus sinicus* (*Calanussinicus*)-*anchovy* (*Engraulis japonicus*)-*Spanish mackerel* (*Scomberomorus Niphonius*)" and others as the main research line, with intention of offering a scientific basis for overcoming the bottleneck the sustainable development of China's marine industry[1]. Nutritional quality is the important content in GLOBEC project[5]. The United Nations FAO and the WHO's report indicates that food protein quality is usually assessed according to amino acid composition and protein digestibility[6]. In recent years, amino acid's effect in ecological system is studied from various perspectives in international academic circle. Jin and Qian[7] studied amino acid's effect on *Hydroides* elegans. Fantle *et al.*[8] did research on the importance of stable isotope of amino acid monomer and animal for *Callinectes sapidus*. Stable nitrogen isotope is widely used in the explanation of marine food chain and few researchers studied on additional factors affecting the change of $\delta^{15}N$ value. Schmidt *et al.*[9] study showed that specific amino acid composition and metabolism of organism interfere the explanation of $\delta^{15}N$ value in nutrition level. Most of the studies by domestic scholars focus on composition and content of amino acid in a variety of marine organisms[10-16] and only a few of them involves food relationship between different organisms[17]. Food relationship is the basic form of marine ecosystem's structure and function, energy flow of food chain is the key biological process for understanding ecosystem resources production and its dynamic change. Amino acid is an important component of complex nutrient circulation of marine ecosystem. Therefore, study on the transfer process of amino acid in lower trophic level of marine food chain, will help to understand nutrient flow mechanism of key species and their conversion process.

## EXPERIMENTAL

**Laboratory incubation experiment:** In order to simulate key species food chain in Yellow sea and East China sea, *Calanus sinicus* was used as live bait for culturing *Engraulis japonicus* and chlorella was used as *Calanus sinicus*'s bait. The experiment was carried out in Mai Island base of Yellow Sea Fisheries Research Institute. Sea water sedimentation and sand filtration were applied. *Chlorella* was cultivated in 1.5 $m^3$ glass steel can in Yellow Sea Fisheries Research Institute with the use of urea and other nutrients. *Chlorella* sample were made of several hundred milliliters of algae liquid, after vacuum filtering, it had to go through Whatman GF/F glass fiber filter. *Calanus sinicus* were obtained through fishing in coastal water, after selected purification, they were cultivated in 22 $m^3$ glass steel pool of sea water with chlorella, the water temperature was controlled at 16-20 °C. At the beginning of experiment, filtered seawater's temperature was low, heating was needed to ensure that the temperature was above 16 °C, with higher temperature, they could be cultivated at room temperature. *Calanus sinicus* was selected with use of self-made mesh. The 16 *Engraulis japonicus* were provided by the researcher Sun Yao from Yellow Sea Fisheries Research Institute, they were 9-10 cm long and had been cultivated in glass steel tank with flowing seawater and artificial bait. The seawater was not different from natural seawater in DO, $NH_4$-N, pH and salinity index. After 2-day domestication, *Calanus sinicus* which had been cultivated artificially were used as bait, the frequency was 2 times every day. In this way, the key species's food chain "*Chlorella-Calanus sinicus-Engraulis japonicus*" was formed. In the controlled experiment, 1 *Engraulis japonicus* sample was collected every 5 days. The fish's feces were collected once a day in the earlier stage of the experiment. During the late of the experiment, because of the decrease in the number of the fish, the feces were collected one two days. A straw was used to collect the stool and remaining bait in the bottom of groove, then they were filtered with 450 °C Whatman GF/F glass fiber filter. Then amino acid analysis was made on collected *Chlorella*, *Calanus sinicus* and *Engraulis japonicus*, feces after vacuum frozen drying. The experiment lasted for 76 days.

**Sample analysis:** Early amino acid quantitative analysis was conducted with the use of liquid chromatography (LC ) or automatic amino acid analyzer[10-17], since the nineteen ninties, gas chromatography- mass spectroscope (GC-MS) was developed to determine composition and content of amino acid[18-20]. On this basis, together with isotope ratio mass spectrometry, gas chromatography (GC-MS) can further determine carbon and nitrogen isotope of single amino acid (GC-C-IRMS), this method has been rapidly developed and applied[21,22].

**Derivatization of samples:** In order to measure composition and content of amino acid with the use of gas chromatography, firstly, it is necessary to derivatize amino acid which has strong polarity and poor thermal stability and strong chemical activity into amino acid ester which has weak polarity, strong volatileness and good thermal stability. The derivatization process[18-21] was as follows: preparing 3 mol/L hydrochloric acid *n*-butanol solution and adding 25 µL acetyl chloride in per ml of the solution. Preparing acid amino acid solution in ampoule bottle, adding 2 mL 3 mol/L hydrochloric acid *n*-butanol solution; Esterifying it at 110 °C for 1 h after sealing. After the reaction is finished, cooling the ampoule bottle to room temperature; Opening the ampoule bottle and vacuum drying at 25 °C, adding 0.5 mL methylene chloride ($CH_2Cl_2$), then evaporating to dryness. Adding 0.25 m of $CH_2Cl_2$ and TFAA into the bottle, acylating in drying box at 110 °C constant temperature for 10 min after sealing the bottle. After the reaction, putting theampoule bottle at room temperature. Opening the ampoule bottle and removing $CH_2Cl_2$ and TFAA with moderate nitrogen. Finally letting reaction products dissolve in 0.5 mL $CH_2Cl_2$ and saving in 4 °C refrigerator.

Amino acid measurement was carried out in Agilent 6890N gas chromatography and 5973N mass spectrometry (GC-MS). HP5-MS colour spectrum column (30 m × 0.32 mm × 0.2 µm) was used. Operating conditions of gas chromatography were as follows[18,19]: gasification chamber temperature: 250 °C; transmission line temperature: 280 °C; beginning temperature of colour spectrum column: 50 °C, the temperature rose to 220 °C with the speed of 20 °C/min and to 280 °C with the speed of 10 °C/min and the temperature remained for 3 min. Splitless injection method was applied. The injection volume was 1 µL. Mass spectrometric conditions: 1200 V voltage multiplier of EI ion source, ion source temperature 230 °C; quadrupole temperature: 150 °C; quality range of full screen scan (SCAN): 33-600 mau. Peak height and peak area of various components were obtained from the chromatogram diagram of total ion flow. The repeated measurement results showed that relative standard deviation of amino acid content for each sample was 3-9 %.

**Data analysis:** A total of 16 *Engraulis japonicus*, 7 *Chlorella* samples, 5 *Calanus sinicus*, 1 synthetic diet and 27 stool of *Engraulis japonicus* were collected. Amino acid composition and content of all samples were analyzed (with sample stem percentage as weight basis). Table-1 shows components of key species food chain, average content of 14 kinds of amino acids for synthetic diets before the experiment.

## RESULTS AND DISCUSSION

**Analysis results on amino acid content of key species food chain:** Analytical results showed that total amino acid content of *Chlorella* was the lowest, only 6.10 %, and *Calanus sinicus* significantly increased the figure to 24.16 % (Table-1). On this basis, as low and middle level fish, *Engraulis japonicus* further increased the figure to 27.06 % and amino acid content of fish feces was only 6.75 %. For comparison, the author analyzed amino acid content of compound feed before the simulation experiment. The results showed that amino acid content of the compound feed was as high as 48.12 %.

**Amino acid composition of various key species:** The main factors affecting protein feed's nutritional value were composition of amino acids and amino acid digestibility. As shown in Table-1, among the measured amino acids (14 kinds), there were 7 were essential amino acids (threonine, valine, leucine, isoleucine, methionine, phenylalanine and lysine) and 7 non-essential amino acids (alanine, glycine, serine, proline, tyrosine and aspartic, glutamic).

Amino acid content of *Chlorella* was relatively low, among the essential amino acids, content of leucine and valine were the richest, while content of lysine and methionine was relatively lower. Among non-essential amino acids, aspartic acid and glutamic acid's content was high, while content of tyrosine and serine was low (Table-1).

Compared with this, in *Chlorella* which was cultivated by Wang *et al.*[16] through ozone treatment of seawater, amino acid content of histidine was the highest, which was followed by glutamic acid, aspartic acid, proline, valine and leucine. Sun[14] measured amino acid content of *Spirulina platensis, Dunaliella salina* and other several marine microalgaes. His results showed that amino acid content of marine microalgaes was as follows: aspartic acid > glycine > alanine. The comparison of these results demonstrated that in phytoplankton, amino acid composition was basically the same, but the specific ranking still had some differences, which may also relevant to their growth conditions to some degree.

Amino acid content of *Calanus sinicus* was as follows: glutamic acid > aspartic acid > lysine > leucine > valine (Table-1). This was in line with Tan *et al.*[17] measurement results of marine copepods' amino acid, their ranking of amino acid content was glutamic acid > aspartic acid > alanine > leucine > lysine > valine. *Calanus sinicus*'s amino acid content has large similarity with *Chlorella*. In the essential amino acids, lysine and leucine was most, while methionine was less. In the non-essential amino acids, they were more close, glutamic acid and aspartic acid' content were high and serine and tyrosine's content was low. One obvious difference was that lysine content of *Calanus sinicus* was significantly higher than that of *Chlorella*. Amino acid content of *Chlorella* and *Calanus sinicus* which is a dominant species of the Yellow sea and East China sea were calculated. The results showed that although total amino acids content of them was different nearly 4 times and there was a significant correlation between content of 14 kinds of amino acids, the correlation coefficient $r = 0.606$ ($p < 0.05$). This was in line with Tan *et al.*[17] research results- amino acid content of phytoplankton copepods in the Gulf of Sanya and the Pearl River Estuary have significant correlation with phytoplankton. However, there was little positive correlation between essential amino acids of *Calanus sinicus* and *Chlorella*, the correlation coefficient $r = 0.443$, which mainly reflects the different in content of lysine; while there was a significant positive relationship among nonessential amino acids, the correlation coefficient $r = 0.856$ ($p < 0.05$). The comparison showed that, as the primary consumer, *Calanus sinicus* played a crucial role in the food chain, it not only transferred plant protein of phytoplankton into animal protein, but also increased amino acid content by nearly 3 times. In terms of amino acid composition, they had certain similarity, in 14 kinds of amino acids, lysine, tyrosine and glycine's amino acid content rose by up to 5 times. Lysine contents increase is very important for human being, this is because lysine in cereal protein of main food is the most important amino acid and rich lysine in seafood can help replenish lysine in plant protein[13], this is closely related to *Calanus sinicus* and other zooplankton's role in nutrition transfer of food chain.

According to Table-1, in essential amino acids of *Engraulis japonicus*, lysine and leucine content were highest, which were, respectively 19 and 2.60 %, while methionine content was relatively low, only 0.56 %. In nonessential amino acids, aspartic acid content was the highest, it was 3.35 %, what was different was that glutamic acid content was only 1.70 %. The lowest two were serine and tyrosine, which also the case for *Calanus sinicus*. By contrast, the most abundant amino acids in other kinds of fishes were glutamate, aspartate, lysine and leucine[10,11].

After comparing them, their kinds of amino acids were exactly the same, there was little different only in the content. Fig. 1 showed that *Engraulis japonicus*'s amino acid content changed over experimental time. Since the *Engraulis japonicus* samples used in the experiment were wild juveniles which

TABLE-1
AMINO ACID PERCENT CONTENTS OF CONSTITUENTS OF KEY SPECIES FOOD CHAIN AND SYNTHESIZED BAIT (%)

| Type of amino acid | | *Chlorella* | *Calanus sinicus* | *Synthetic* bait | Flesh of *anchovy* | Feces |
|---|---|---|---|---|---|---|
| Essential amino acid | Thr | 0.43 | 1.19 | 2.20 | 1.26 | 0.37 |
| | Val | 0.63 | 2.03 | 2.23 | 1.85 | 0.71 |
| | Leu | 0.85 | 2.31 | 4.55 | 2.60 | 0.47 |
| | Iie | 0.29 | 0.99 | 1.18 | 1.06 | 0.23 |
| | Met | 0.16 | 0.39 | 2.03 | 0.56 | 0.04 |
| | Phe | 0.45 | 1.32 | 3.16 | 1.53 | 0.24 |
| | Lys | 0.13 | 2.87 | 5.79 | 6.19 | 0.13 |
| | Total | 2.94 | 11.1 | 21.14 | 15.05 | 2.19 |
| Non-essential amino acid | Ala | 0.44 | 1.57 | 2.84 | 1.57 | 0.52 |
| | Gly | 0.33 | 1.66 | 2.71 | 1.37 | 0.92 |
| | Ser | 0.31 | 0.99 | 2.27 | 1.11 | 0.41 |
| | Pro | 0.45 | 1.49 | 3.80 | 1.76 | 0.57 |
| | Asp | 0.75 | 2.87 | 6.53 | 3.35 | 0.73 |
| | Tyr | 0.17 | 1.44 | 2.16 | 1.17 | 0.63 |
| | Glu | 0.71 | 3.03 | 6.67 | 1.70 | 0.78 |
| | Total | 3.16 | 13.05 | 26.98 | 12.03 | 4.56 |
| Sum total | | 6.10 | 24.16 | 48.12 | 27.06 | 6.75 |

TABLE-2
CORRELATION COEFFICIENTS BETWEEN AMINO ACID CONTENTS OF DIFFERENT BAITS AND *anchovy*

| Type of bait | *Synthetic* bait | | | *Calanus sinicus* | | |
|---|---|---|---|---|---|---|
| Type of amino acid | 14 types of amino acid | Essential amino acid | Non-essential amino acid | 14 types of amino acid | Essential amino acid | Non-essential amino acid |
| Anchovy R2 | 0.726** | 0.803* | 0.740 | 0.580* | 0.449 | 0. 772* |
| Anchovy R16 | 0.357 | 0.171 | 0.768* | 0.692** | 0.662 | 0.859* |

**Correlation is significant to a 0. 01 significant level, *Correlation is significant to a 0. 05 significant level

had been cultivated with synthetic bait for about half a year, amino acid content of the compound feeds was up to 48.12 %, after the start of the experiment, the bait was replaced with *Calanus sinicus* (its amino acid content was 24.16 %). In Fig. 1, the slope of the regression line was -0.0603, which indicated that amino acid content of *Engraulis japonicus*'s muscle declined over time, which suggest that the bait had obvious effect on amino acid content of *Engraulis japonicus*'s muscle.

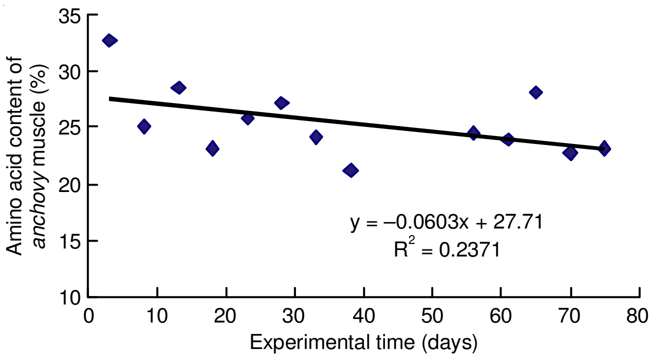


Fig. 1. Variations in amino acid content of anchovy muscle with experimental time

Comparing amino acid content of *Engraulis japonicus* samples R2 and R16 with 2 different baits-compound feed and *Calanus sinicus*, the results were shown as Table-2. The obvious correlation between *Engraulis japonicus* sample R2 and compound feed ($r = 0.726$, $p < 0.01$) proved that there had been nutrient balance between *Engraulis japonicus* and compound feed before the experiment. After 76 days of cultivating *Calanus sinic*, there was significant positive correlation between amino acid content of the sample R16 and *Calanus sinicus* at the end of the experiment, correlation coefficient $r = 0.692$ ($p < 0.01$); while its correlation with amino acid content of compound feed became weak, the correlation coefficient $r = 0.357$. It further demonstrated that change of bait had significant influence on amino acid content of *Engraulis japonicus*. Huang *et al.*[10] also found that in natural waters and pond environment, there was certain different in amino acid content of *Calanus sinicus*'s muscle. They thought this was mainly related to feed, natural bait could make *Calanus sinicus* have higher amino acid content. In addition, it was also related to gender, the female had slightly higher content than the male. The content would also decline in the state of starvation. We can find that amino acid content of *Engraulis japonicus*'s muscle were affected by many aspects. Statistical calculations of amino acid also indicated that, for the essential amino acids, there was also a significant positive correlation between R2 sample and compound see, the correlation coefficient $r = 0.803$ ($p < 0.05$); while there was a positive correlation between R16 sample and *Calanus sinicus*, the correlation coefficient $r = 0.662$, there was no obvious correlation between R16 and synthetic bait, the correlation coefficient $r = 0.171$, which showed same regularity. As for the nonessential amino acids, there was a positive correlation between *Engraulis japonicus* sample and synthetic bait, *Calanus sinicus*, the correlation coefficient was 0. 740-0. 859. This indicated that the essential amino acids were more dependent on the bait than non-essential amino acids. Japanese scientists tested effects of feeding conversion on soleus, they studied the composition of stable carbon and nitrogen isotopic and obtained useful results[23].

**Transfer of amino acids in the process of metabolism:** In the experiment, the relationship between *Engraulis japonicus* and amino acid content of other feces. In the collected feces, valine and leucine contents were high, while methionine and lysine content were low. In contrast, in *Engraulis japonicus* sample, lysine and leucine contents were high, this indicated that in the process of metabolism, absorption rate of lysine was relatively high, while absorption rate of valine was relatively low. The results of SPSS statistics calculation showed that there was no significant correlation between average amino acid content and average value of feces's amino acid content, the correlation coefficient r was only 0.399, the correlation coefficient of 7 essential amino acids was slightly higher, which was 0.529, while the correlation coefficient of 7 nonessential amino acids was only 0.221. There was only a weak correlation between amino acid content of *Calanus sinicus* and *Engraulis japonicus*'s faces, correlation coefficient of all amino acids r was 0.508, the coefficient for 7 essential amino acids was 0.496 and the coefficient for 7 kinds of non-essential amino acids was 0.590. This indicated that amino acid content of *Engraulis japonicus* faces was mainly determined by its metabolism progress, it had a little correlation with *Calanus sinicus*'s amino acid content.

## Conclusions

• In *Engraulis japonicus* food chain, *Calanus sinicus* played a vital role in the transmission of amino acids, it not only transferred plant protein into animal protein, but also greatly improved lysine content and protein quality, which had a important role in avoiding the shortage of lysine in plant protein and this was a key link in amino acid transfer in the whole food chain.

• According to the change of amino acid content in food conversion of *Engraulis japonicus*, physiological process was a decisive factor for amino acid content, but it was not the only one, amino acid content of bait also had significant impact on amino acid content of *Engraulis japonicus*'s muscle.

• *Engraulis japonicus* was a key species in Yellow sea and East China sea, it had great impact on economic species resources, amino acid content of its faeces was mainly determined by its metabolism progress, there was weak correlation between it and amino acid content of bait.

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