Differences on lipid quality index and amino acid profiles of European Anchovy caught from different area in Turkey

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**Abstract**

**Introduction.** European anchovy is a nutritive food preferred by everybody with high omega 3 contents.

**Materials and methods.** Within the scope of the study, the anchovy which was caught from different seas in Turkey were compared in view of its lipid quality index and amino acid (AA) profile.

**Result and discussion.** The amount of SFA, MUFA and PUFA’s of the Aegean Sea (AS), Black Sea (BS), and the Marmara Sea (MS) anchovy were 41.31, 11.18, 48.10; 32.38, 31.39, 36.24% and 32.92, 32.24, 34.82, respectively. The SFA and PUFA in the AS anchovy were found higher than the others (p<0.05). Similarly, the highest DHA contents were found as 29.51% in the AS anchovy (p<0.05). The oleic acid of BS anchovy were more than 3 times of the AS anchovy. Glutamic acid was the highest essential AA in the groups. The lysine, aspartic acid, alanine, glycine, glutamic acid, isoleucine, leucine, methionine, phenylalanine, proline in the AS anchovy were higher than the other groups. The histidine of the anchovies was similar (p>0.05). The ratio of EAA with non-EAA of the AS, BS and MS anchovies were found to be 1.21, 1.31 and 1.21 respectively.

**Conclusion.** It has been found that anchovy contains high amounts of essential fatty acids and EAAs. Especially the AS anchovy was higher quality in view of EPA+DHA and total EAAs compositions.
Introduction

With increasing demand for quality food throughout the world, the trend towards fish meat has increased. Fish is a nutritive food preferred by young and old people with high protein, mineral matter, water, and unsaturated fat content.

One of the favorite sea foods that can be caught almost all seasons in the territorial waters of our country is the anchovy. European Anchovy (Engraulis encrasicolus Linnaeus, 1758) is a pelagic fish species which is preferred by consumers because of its price and taste. Anchovy which is caught during fishing season (September 1- April 15). European anchovy in Turkey is the most important species both in weight and in a value of landings. Annual anchovy caught after the 2000 year in Turkey peaked in the 2001s at over 257000 tons but, since then, landings gave declined, averaging 74304 tons for the last 3 years (2015-2017). In 2017, 322.173 tones fish were caught in Turkey land waters. 49% of all fish caught in our seas were anchovy. 133.766 tons of anchovy were caught from the Black Sea and the others were caught from the Aegean Sea > The Marmara Sea > The Mediterranean Sea. In 2017, the average selling price of 1-kilo anchovy was 5.5TL [1].

In this study, it was investigated the fatty acids, lipid quality index and amino acid contents of the anchovy which were caught from different seas in Turkey.

Material and methods

The study was carried out in December 2017 with the Anchovy (Engraulis encrasicolus Linnaeus 1758) caught from the Aegean Sea, Black Sea and the Marmara Sea in Turkey. For sampling, twelve kilos fish were obtained from the fisheries at the same time from different regions. The fish were transported to the laboratory under cold storage conditions. And then the fish were filleted and analyzed.

Amino acid analyzes and fatty acids composition were performed after digestion derivatization method according to HPLC pre-column [2] and IID-19 method [3], respectively. Amino acid profiles of the anchovy were calculated as total essential amino acid (ΣEAA) including Histidine (His), Threonine (Thr), Arginine (Arg), Valine (Val), Methionine (Met), Phenylalanine (Phe), Isoleucine (Ile), Leucine (Leu), Tyrosine (Tyr) and Lysine (Lys); total non-essential amino acid (ΣNEAA) including Aspartic acid (Asp), Glutamic acid (Glu), Serine (Ser), Glycine (Gly), Citrulline (Cit), Alanine (Ala) and Proline (Pro); total sweet amino acids (Σ Sweet AA) including Glu, Ser, Gly and Ala; total bitter amino acids (Σ Bitter AA) including His, Arg, Met, Phe, Lys according to Schiffman [4] and Boisen, et al. [5].

Lipid quality indexes of lipid (Atherogenicity index-Al, Thrombogenicity index-TI [6], flesh lipid quality index (FLQ index [7] and hypocholesterolaemic/hypercholesterolaemic ratio (Hh) [8] following formulas were used;

\[\text{AI} = \frac{[(C12:0 + 4\times C14:0) + (C16:0)]}{[(\Sigma n6 + \Sigma n3 + \Sigma MUFA)]}\]
\[\text{TI} = \frac{[(C14:0 + C16:0 + C18:0)]}{[(0.5 \times \Sigma MUFA) + (0.5 \times \Sigma n6) + (3 \times \Sigma n3) / (\Sigma n6)]}\]
\[\text{FLQ} = 100 \times \frac{[(EPA\%+DHA\%)]}{(Total fatty acids\%)\}}\]
\[\text{Hh} = \frac{(C18:1n-9 + C18:2n-6 + C20:4n-6 + C18:3n-3 + C20:5n-3 + C22:5n-3 + C22:6n-3) / (C14:0+C16:0)\}}\]

All analyzes were performed in 2 replicates with 3 parallel. The data obtained at the end of the study were evaluated with one-way ANOVA using Minitab Release 17 package program and Tukey test was used for determination of the significance level of differences in-groups and between groups [9]. Figures and schedules are prepared using MS Office 2010 software.
Results and discussion

The crude protein, crude fat contents of the Aegean, Black Sea and the Marmara Sea anchovies analyzed were 20.16±0.29, 0.74±0.01; 18.75±0.30, 9.33±0.75 and 18.80±0.12 g/100g – 11.76±0.61 g/100g, respectively.

Fatty acids composition of European anchovy oil

The fatty acid composition results of anchovy oil caught from different seas were in Table 1.

Table 1

| Fatty acid composition of Anchovy caught from different seas (%) | Aegean Sea Anchovy | Black Sea Anchovy | Marmara Sea Anchovy |
|---|---|---|---|
| **Mean** | **Std. Error** | **Mean** | **Std. Error** | **Mean** | **Std. Error** |
| Butyric acid (C4:0) | 8.28 | 0.03a | 2.87 | 0.49b | 2.89 | 0.19b |
| Caproic acid (C6:0) | 0.01 | 0.00a | 0.00 | 0.00b | 0.00 | 0.00b |
| Caprylic acid (C8:0) | 0.00 | 0.00a | 0.00 | 0.00a | 0.00 | 0.00a |
| Capric acid (C10:0) | 0.03 | 0.00a | 0.01 | 0.00a | 0.04 | 0.02a |
| Undecanoik acid (C11:0) | 0.01 | 0.00a | 0.01 | 0.00a | 0.01 | 0.01a |
| Lauric asid (C12:0) | 0.13 | 0.00b | 0.13 | 0.00b | 0.31 | 0.02a |
| Tridecanoic acid (C13:0) | 0.13 | 0.00b | 0.11 | 0.00b | 0.25 | 0.01a |
| Myristic asid C14:0 | 5.63 | 0.00b | 6.07 | 0.02a | 3.84 | 0.17c |
| Penta decanoic acid C15:0 | 1.59 | 0.00b | 1.84 | 0.04b | 2.95 | 0.18a |
| Palmitic acid C16:0 | 20.24 | 0.74a | 15.41 | 0.10b | 14.91 | 0.89b |
| Heptadecanoic acid C17:0 | 1.89 | 0.01b | 1.85 | 0.01b | 2.68 | 0.16a |
| Stearic acid C18:0 | 0.01 | 0.00a | 0.00 | 0.00b | 0.00 | 0.00b |
| Arachidic acid C20:0 | 0.22 | 0.00c | 0.72 | 0.00b | 1.00 | 0.06a |
| Heneicosenoic acid C21:0 | 0.05 | 0.00c | 0.14 | 0.00b | 0.19 | 0.01a |
| Behenic asid C22:0 | 2.62 | 0.01ab | 2.23 | 0.04b | 2.80 | 0.20a |
| Tricosanoic acid C23:0 | 0.15 | 0.00b | 0.27 | 0.00a | 0.06 | 0.00c |
| Lignoseric acid (C24:0) | 0.33 | 0.01c | 0.71 | 0.01b | 1.03 | 0.07a |
| **SFA** | **41.31** | **0.80a** | **32.38** | **0.44b** | **32.92** | **0.16b** |
| Myristoleic acid (C14:1) | 0.36 | 0.01b | 0.55 | 0.01b | 1.40 | 0.08a |
| Penta decanoic acid (C15:1)cis-10- | 0.20 | 0.00c | 0.29 | 0.01b | 0.55 | 0.01a |
| Palmitoleic acid C16:1n7 | 0.37 | 0.00c | 1.72 | 0.01a | 0.84 | 0.03b |
| Heptadecanoic acid C17:1cis -10- | 0.42 | 0.01b | 1.19 | 0.00a | 1.21 | 0.08a |
| Oleic acid C18:1n9c | 4.33 | 0.02c | 14.81 | 0.16a | 10.59 | 0.71b |
|                         | Aegean Sea Anchovy | Black Sea Anchovy | Marmara Sea Anchovy |
|-------------------------|-------------------|-------------------|---------------------|
|                         | Mean   | Std. Error | Mean   | Std. Error | Mean   | Std. Error |
| Elaidic acid (C18:1n9t) | 0.22   | 0.01c     | 6.04   | 0.16b     | 8.90   | 0.57a      |
| Eicosenoic acid C20:1 cis 11 | 1.73  | 0.01c     | 2.87   | 0.02b     | 3.54   | 0.29a      |
| Erucic acid (C22:1n-9)  | 0.82   | 0.03c     | 1.94   | 0.04b     | 2.88   | 0.18a      |
| Nervonic acid C24:1n-9  | 2.75   | 0.00a     | 1.99   | 0.00b     | 2.35   | 0.16b      |
| **MUFA**                |        |           |        |           |        |            |
| Linoleadic acid C18:2n6t | 11.18  | 0.07b     | 31.39  | 0.27a     | 32.24  | 2.09a      |
| Linoleic acid (LA) C18:2n6c | 3.19  | 0.02c     | 4.12   | 0.02b     | 5.09   | 0.32a      |
| Alpha-Linolenic acid (ALA) C18:3n3 | 0.68  | 0.01b     | 2.10   | 0.01a     | 0.00   | 0.00c      |
| Gamma-Linolenic acid (GLA) C18:3n6 | 1.07  | 0.02c     | 2.78   | 0.01b     | 3.68   | 0.25a      |
| Eicosadienoic acid C20:2 cis 11.14 | 0.67  | 0.00b     | 0.88   | 0.00b     | 1.39   | 0.10a      |
| Arachidonic acid C20:4n6 | 2.62   | 0.01ab    | 2.23   | 0.04b     | 2.80   | 0.20a      |
| Eicosatrienoic acid C20:3n6 cis-8.11.14 | 0.00  | 0.00c     | 1.12   | 0.01b     | 1.34   | 0.09a      |
| Eicosatrienoic acid C20:3n3 cis-11.14.17 | 0.00  | 0.00a     | 0.00   | 0.00a     | 0.00   | 0.00a      |
| Docosapentaenoic acid (DPA) (C22:5n3) cis-7.10.13.16.19 | 0.00  | 0.00a     | 0.00   | 0.00a     | 0.00   | 0.00a      |
| Eicosapentanoic acid (EPA) C20:5n3 cis 5.8.11.14.17 | 10.08 | 0.09a     | 8.34   | 0.02a     | 4.37   | 4.37a      |
| Docosadienoic acid C22:2 cis13.16 | 0.15  | 0.00b     | 0.27   | 0.00a     | 0.30   | 0.02a      |
| Docosahexanoic acid (DHA) C22:6n-3 cis-7.10.13.16.19 | 29.51 | 0.42a     | 12.26  | 0.08b     | 12.11  | 0.88b      |
| **PUFA**                |        |           |        |           |        |            |
|                         | 48.10  | 0.27a     | 36.24  | 0.17b     | 34.82  | 2.26b      |
| n-3                     | 40.27  | 0.33a     | 22.70  | 0.09b     | 16.48  | 3.49b      |
| n-6                     | 5.96   | 0.04c     | 9.60   | 0.08b     | 12.98  | 0.86a      |
| **TOTAL**               | 100.58 | 0.59a     | 100.01 | 0.00a     | 99.98  | 0.00a      |
| Undefined               | -0.58  | 0.59a     | -0.01  | 0.00a     | 0.03   | 0.00a      |
| n-3/n-6                 | 6.76   | 2.37      | 1.29   |           |        |            |

Means with different lowercase letters in the same line are significantly different (p<0.05) from groups. p<0.05
The amount of SFA (saturated fatty acids), MUFA (monounsaturated fatty acids) and PUFA (polyunsaturated fatty acids) of anchovy was max. 41.31% (in the Aegean Sea anchovy), max. 32.24% (in the Marmara Sea anchovy) and max. 48.10% (in the Aegean Sea anchovy), respectively (Figure 1). A large amount of SFA content is composed of palmitic acid (max 20.24% in the Aegean Sea anchovy), butyric acid (max 8.28% in the Aegean Sea anchovy), myristic acid (max 6.07% in Black Sea anchovy) and behenic acid (max 2.80% in the Marmara Sea anchovy). While about 47% of the MUFAs composed of oleic acid in Black Sea anchovy. The predominant n3 fatty acids found in fish meat is especially DHA. Similarly, present study; Özden [10] and Kocatepe and Turan [11] were reported the predominant SFA, MUFA, and PUFA’s in the Marmara Sea anchovy were; palmitic acid, oleic acid, and EPA-DHA, respectively.

Fish fats contain 20-30% of saturated fatty acids, 70-80% of unsaturated fatty acids [12, 13]. In the present study the fatty acids content of anchovies was compared, it was seen that the amount of SFA, MUFA and PUFA were changed between 32% - 42%, 11% - 33% and 34% - 49%, respectively.

The SFA contents of the Aegean Sea anchovy was highest (p>0.05). Researchers reported the different SFA ratios were 40.77% [14] in the Marmara Sea anchovy; 38.62, 35.20 and 33.57% in November, December and January in the Black Sea anchovy [15], 37.91 to 34.04% (from October to April) in the Black Sea anchovy [16]; 42.2% in the Black sea and the Marmara Sea [17], 36.23% in the Marmara Sea anchovy [10] and 37.81% [18].

Most of the n-6 fatty acids form was linoleic acid and arachidonic acid in all groups. The n-3/n-6 ratio is the best index for comparing relative nutritional values of fish oils from

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Figure 1. The total fatty acids composition of anchovy (%).
different species [19]. In the study, the maximum n-3/n-6 proportional value was determined as 6.76 in the Aegean Sea anchovy, 2.37 in Black sea anchovy and 1.29 in the Marmara Sea anchovy, respectively.

The minimum value of the PUFA/SFA ratio recommended is 0.45 [20]. PUFA/SFA ratio of anchovy was higher than the limit value in all groups (Table 2).

Table 2

| PUFA/SFA ratio, lipid quality indices, crude lipid and EPA+DHA value (to meet the EPA and DHA recommendation) of whiting meat and roe (%) | Aegean Sea Anchovy | Black Sea Anchovy | Marmara Sea Anchovy |
|---|---|---|---|
| PUFA/SFA | 1.17 | 1.13 | 1.06 |
| AI | 0.75 | 0.62 | 0.49 |
| TI | 0.19 | 0.24 | 0.26 |
| FLQ | 39.37 | 16.48 | 19.01 |
| h/H | 1.98 | 2.51 | 2.81 |
| Crude lipid% | 0.74 | 11.76 | 9.33 |
| EPA+DHA | 0.29 | 2.42 | 1.54 |

Flesh Lipid Quality Indices (FLQ) indicate the global dietetic quality of lipids and their potential effects on the development of coronary disease [21]. The FLQ values of the Aegean Sea anchovy was highest. AI and IT indexes should be low to prevent cardiovascular diseases related with lipid intake [6] and the high value of h/H ratio represents high-quality lipids. In this study, AI and IT indexes were measured to be lower than 1.0 but high in h/H in all groups. Ouraji et al. [22] and Stancheva et al. [23] determined that higher values of AI and IT (>1.0) are detrimental to human health. Especially the h/H ratio of the Marmara Sea anchovy was higher than the others. It was showed that the lipids quality of the Aegean Sea anchovy had the highest quality in view of the lipid quality.

The ratio of n-3/n-6 fatty acid is commonly used as an index for assessing the nutritional quality of fishery products [24]. The ratio of n-3/n-6 of the Aegean Sea anchovy was the highest (6.76). The maximum amount of n3 and n6 were detected in the Aegean Sea anchovy and the Marmara Sea anchovy, respectively. Several studies report that a high n-6/n-3 FA ratio is associated with an increased risk of CRC (colorectal cancer), PC (Pancreatic cancer) and BC (breast cancer). Since a risk associated with n-6 has not been demonstrated, it can be concluded that a low n-3 PUFA intake is responsible for the observation [25]. The difference in the fatty and fatty acid composition of seafood depends on different factors such as nutritional pattern, geographical conditions, environmental temperature, season, hunting area, fish size, sex and species [13, 26].

For adult pregnant and lactating females, the minimum intake for optimal adult health and fetal and infant development is 0.3 g/d EPA+DHA, of which at least 0.2 g/d should be DHA [25]. Similarly, the British Nutrition Foundation [27] has emphasized that people who care for balanced and healthy nutrition should get 0.2 g of EPA + DHA every day. Daily intake of 100 g anchovy consumption (caught from all sea studied in this study) is meet the EPA + DHA requirement. This study was indicating that the very oily Black sea anchovy has more EPA+DHA than the others. Especially the crude fat value of the Aegean Sea anchovy was very low, for this reason to meet the EPA and DHA recommendation was lowest according to the BNF.
Amino acids composition of European anchovy

The results of the amino acid compositions of the anchovies caught from different seas were shown in Table 3. Total nine essential amino acids except from tryptophan and nine non-essential amino acids were identified in the anchovy. The maximum amount of glutamic acid detected in the Aegean Sea anchovy (p<0.05). The content of aspartic acid in the Black Sea and the Marmara Sea anchovies were found to be similar (p>0.05).

The highest amount of essential amino acids was detected for lysine in all groups. The amount of lysine was lowest in the Marmara Sea anchovy. The ratio of essential amino acids to non-essential amino acids (EA/NEA) in fish meat was found to be 1.21 in the Aegean and Marmara Sea anchovy and 1.31 in Black Sea anchovy.

| Table 3 | Amino acid composition of anchovy caught from different Sea (mg/kg). |
|---------|---------------------------------------------------------------|
|         | Aegean Sea Anchovy | Black Sea Anchovy | Marmara Sea Anchovy |
|         | Mean       | Std. Error  | Mean       | Std. Error  | Mean       | Std. Error  |
| Alanine | 1.68       | 0.02a      | 1.35       | 0.04b      | 1.24       | 0.01b      |
| Arginine* | 1.53      | 0.01a      | 1.37       | 0.05a      | 1.17       | 0.02b      |
| Aspartic Acid | 2.39   | 0.07a      | 1.88       | 0.03b      | 1.85       | 0.04b      |
| Cysteine | 0.32       | 0.00a      | 0.31       | 0.01a      | 0.24       | 0.00b      |
| Glutamic Acid | 3.35   | 0.04a      | 2.92       | 0.02b      | 2.47       | 0.02c      |
| Glycine | 1.05       | 0.01a      | 0.59       | 0.01b      | 0.83       | 0.03c      |
| Histidine* | 1.11      | 0.01a      | 0.93       | 0.09a      | 0.83       | 0.06a      |
| Isoleucine* | 0.93     | 0.02a      | 0.78       | 0.02b      | 0.70       | 0.03b      |
| Leucine* | 1.88       | 0.01a      | 1.56       | 0.05b      | 1.43       | 0.00b      |
| Lysine* | 2.95       | 0.15a      | 2.80       | 0.12ab     | 2.24       | 0.04b      |
| Methionine* | 0.80     | 0.03a      | 0.58       | 0.01b      | 0.62       | 0.02b      |
| Ornithine | 0.06       | 0.01a      | 0.07       | 0.01s      | 0.06       | 0.01a      |
| Phenylalanine* | 1.16   | 0.00a      | 0.98       | 0.02b      | 0.91       | 0.00c      |
| Proline | 0.99       | 0.01a      | 0.81       | 0.00b      | 0.77       | 0.01b      |
| Serine | 1.19       | 0.03a      | 1.03       | 0.07s      | 0.94       | 0.06a      |
| Threonine* | 1.27      | 0.04a      | 1.13       | 0.06sb     | 0.97       | 0.00b      |
| Tyrosine | 0.93       | 0.04a      | 0.76       | 0.04s      | 0.75       | 0.03a      |
| Valine* | 1.30       | 0.03a      | 1.31       | 0.02s      | 1.03       | 0.01b      |
| Taurine | 0.38       | 0.02a      | 0.32       | 0.00s      | 0.35       | 0.01a      |
| Total amino acids | 25.26 | 0.31a      | 21.46      | 0.25b      | 19.39      | 0.15c      |
| Total Essential amino acids (EA) | 13.86 | 0.15a      | 12.18      | 0.14b      | 10.64      | 0.14c      |
| Total non-essential amino acids(NEA) | 11.41 | 0.15a      | 9.27       | 0.11b      | 8.75       | 0.01b      |
| Sweet amino acids | 7.28   | 0.10a      | 5.88       | 0.07b      | 5.48       | 0.02b      |
| Bitter amino acids | 7.55   | 0.13a      | 6.66       | 0.18b      | 5.76       | 0.12c      |
| EA/NEA | 1.21       | 1.31       | 1.21       | 1.21       | 1.21       | 1.21        |
Fish meat is a protein source with high nutritive value. In fish meat containing all essential amino acid as aspartic acid, glutamic acid and lysine [28]; aspartic acid and glutamic acid are important amino acids that play a role in enzyme activation, preservation of the solubility and ionic character of proteins. One of the most important sources (50-85%) of non-protein nitrogen in fish meat is free amino acids. Free amino acids give the fish meat a characteristic taste.

The level of total sweet AA components was higher in the Aegean Sea anchovy (p<0.05) than the other anchovies, total bitter AA was also higher than when compared the other groups. Proteins are composed of 20 different amino acids. Most proteins contain glutamate in high content. For example, glutamate contents of casein in milk, gluten in wheat, glycine in soybean, and myosin in muscle are 21–35%. Although free glutamate has umami taste, glutamate in proteins has no taste. Proteolysis during fermentation produces free glutamate in high content [29]. Umami taste is found in foods rich in glutamates such as fish, meat, milk, tomatoes, and some vegetables. In addition, the umami flavor is enriched with certain ribonucleotides (inosine and guanosine nucleotides) found in some meats and fish [29-31]. Foods rich in glutamate content, when combined with ribonucleotides, have a higher taste intensity than the sum of both contents [30, 31]. An important component of umami taste is glutamic acid. Glutamate is an amino acid that is very common in foods [32]. The glutamic acid contents of the Aegean Sea anchovy were highest. It can be said that; the umami taste of the Aegean Sea anchovy is more dominant.

The World Health Organization’s [33] on protein and amino acid requirements for human nutrition reports an adult daily protein intake of 0.83g/kg, with essential amino acids; leucine 59 mg/g, lysine 45 mg/g, isoleucine 30 mg/g, threonine 23 mg/g and methionine 16 mg/g. In the present study, about 80 g anchovy meat daily consumption meets the leucine, lysine, isoleucine, threonine and methionine requirements. In addition, 320 g anchovy consumption enough for daily protein requirements (Table 4).

Table 4

| Recommended Daily Intake* | Aegean Sea Anchovy | Black Sea Sea Anchovy | Marmara Sea Anchovy |
|---------------------------|-------------------|-----------------------|---------------------|
| **Protein**               | 60 g **           | 291 g                 | 319 g               | 320g                      |
| **Leucine**               | 59 mg/g           | 64.5g                 | 77.7g               | 71.3g                     |
| **Lysine**                | 45 mg/g           | 31.05g                | 37.8g               | 30.2g                     |
| **Isoleucine**            | 30 mg/g           | 66.3g                 | 80.7g               | 72.4g                     |
| **Threonine**             | 23 mg/g           | 37.3g                 | 44.5g               | 38.2g                     |
| **Methionine**            | 16 mg/g           | 41.1g                 | 48.6                | 51.7g                     |

Conclusion

Fatty acid and amino acid composition of anchovy caught from different seas in Turkey are different from each other. In generally the European anchovy is a good source of n-3 PUFA. Especially the maximum amount of n3 and n6 have detected in the Aegean Sea anchovy and the lipids quality of the Aegean Sea anchovy had the highest quality in view of the lipid quality index. But the crude lipid composition of Black Sea anchovy was highest, for this reason, Black sea anchovy has more EPA+DHA than the others. Especially the crude fat value of the Aegean Sea anchovy was very low, and to meet the EPA and DHA recommendation was lowest according to the BNF. The amino acid composition of anchovies was different from each other, about 80 g European anchovy meet the daily consumption requirements of the leucine, lysine, isoleucine, threonine, and methionine.
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