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RESEARCH ARTICLE

Determination of Amino Acids Composition in Different Tissues of Whiting, *Merlangus merlangus euxinus* (Nordmann, 1840) from the Black Sea, Turkey

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ABSTRACT

LC-MS/MS was used to determine the amino acid composition in muscle, ovary and liver of whiting, *Merlangus merlangus euxinus*, caught off the coast of Sinop province in the Black Sea. A total of 19 amino acids (AA) were found in the different samples. The essential amino acids (EAA) in the different tissues of whiting were 55.9% in the meat, 54.8% in the ovary and 51.7% in the liver of the total amino acids. The AA contents except for Arg, Glu, Pro and Tau in meat and ovary of whiting were not significantly different ($P \geq 0.05$), but the AA contents except for Cys, Tyr, Asp, Orn and Tau of these two tissues were significantly ($P < 0.05$) higher than the AA contents of the liver. These results showed that whiting ovaries have approximately as much protein as the meat.

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Introduction

Whiting, *Merlangus merlangus euxinus* (Nordmann, 1840), is one of the most important commercial demersal fish species in the Black Sea off the coast of Turkey and mainly fished by bottom trawl during autumn and winter and by gillnets throughout the year (Bilgin *et al.*, 2012). In the Black Sea reproduction activity of this species continues during the year and intensive spawning occurs at last three times a year: at the end of summer, in mid-autumn and in early winter (Bilgin *et al.*, 2012). Mazlum and Bilgin (2014) reported that food consumption was intense during spring and summer. The meat of this fish species is consumed locally throughout the year usually by cooking in oil (personal observation).

Seafoods are valuable sources of protein, fatty acids, minerals and vitamins (Tilami and Sampels, 2017). The taste of fish meat is closely related to the biochemical composition especially the protein content (Tilami and Sampels, 2017; Doğan and Ertan, 2017) and the biochemical composition of fish meat can be affected by water temperature and nutrients in the environmental (Doğan and Ertan, 2017). It was reported that the chemical composition of different fish species depends on variables such as season, sexual maturity,

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reproduction time and the nutrient cycle (Limin *et al*., 2006; Özden and Erkan, 2011; Doğan and Ertan, 2017).

Amino acids AA may also be responsible for some of the taste and flavor of fish (Doğan and Ertan, 2017). Fish meat is also considered to be a good source of essential amino acids (EAA), i.e., Arg, Cys, His, Iso, Leu, Lys, Met, Phe, Thr, Tyr and Val. They also contain measureable amounts of Orn and Tau which are not found in proteins (Kim and Lall, 2000; Limin *et al*., 2006; Adeyeye, 2009; Erkan *et al*., 2010ab; Özden and Erkan, 2011; Doğan and Ertan, 2017). It was reported that in fish, 50-80% of the non-protein nitrogenous compounds are AA and significant amounts of these AA are Glu, Arg, Lys, Pro and Tau (Ruiz-Capillas and Moral, 2001; Doğan and Ertan, 2017). Gln is a α-amino acid that is used in the biosynthesis of proteins and its side chain is similar to that of Glu, except the carboxylic acid group is replaced by an amide (Tapiero *et al*., 2002; Watford, 2015). It is classified as a charge-neutral, polar amino acid. Both glutamate and glutamine (Glx) are not considered essential amino acids but they play important roles in maintaining growth and health in both neonates and adults (Watford, 2015). Their chemical characteristics are very similar as well. Both contain nitrogen, belong to a carboxylic acid chemical group, and both glutamate and glutamine are alkaline. Glutamine and glutamate with Pro, His, Arg and Orn comprise 25% of the dietary amino acid intake and constitute the glutamate family of amino acids, which are disposed of through conversion to glutamate (Tapiero *et al*., 2002).

The AA contents different fish species' muscles have been studied using high pressure liquid chromatography (HPLC) (Antoine *et al*., 1999; Kinm and Lall, 2000; Limin *et al*., 2006; Adeyeye, 2009; Erkan *et al*., 2010a; Erkan *et al*., 2010b; Özden and Erkan, 2011; Doğan and Ertan, 2017. Although the fish liver and gonads (especially ovary) are important internal organs, they are not generally consumed. Note that: the ovaries when available of whiting are cooked in oil and consumed by people living in the Black Sea region (personal observation).

In previously studies, there is no investigation related to amino acids profile of different tissues of fish species in the Black Sea. Moreover, LC-MS/MS (Liquid Chromatography Mass Spectrometer) device is able to separate, identify and quantify the requested substance in a mixture at an advanced level (Anonymous, 2016) and in this study we used firstly the Using LC-MS/MS was used to determine the amino acid composition of the muscle, ovary and liver of whiting (*Merlangus merlangus euxinus*) caught along the Sinop coast of the Black Sea.

Materials and Methods

Samples

A total of 3 kg newly caught whiting in the Black Sea were obtained from local fishermen in February 2018 in the Sinop region. Newly caught whiting specimens were brought to the laboratory in ice. The total length of each whiting was measured with a sensitivity of 1 mm. Specimen, gonad and liver wet weights were obtained using a balance with a sensitivity of 0.001 g. The average total length and wet weight of whiting used were 14.0±0.2 cm and 18.7±0.7 g, respectively. The average Gonodosomatic index, GSI = [(gonad weight / total fish weight) x 100)] and the average Hepatosomatic index, HSI = [(liver weight / total fish weight) x 100)] were 4.9±0.4% for GSI (Note that the maturity stages of the ovary used included stage I, stage II and stage III ovaries with stage III being the most/least mature according to Bowers (1954) and 4.2±0.2% for the HSI. After the length and weight determination, the whole edible muscle and liver of both males and females and ovary of females was minced and homogenized using homogenizer and than stored in a freezer at -20ºC for 3 days.

Determination of Amino Acids

The amino acids analyzes of the samples were made in duplicate using the SUBITAM's (Sinop University Scientific and Technological Researches Application and Research Center) Agilent Infinity 1260 HPLC system consisting of a binary pump, a degasser and autosampler coupled with 6460 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA).

For the measurement of the amino acid concentrations, samples were prepared by using Jasem LC-MS/MS amino acid kit (Sem Laboratuvar Cihazları Pazarlama San. ve Tic. Inc. Istanbul/TURKEY) which included five standards for calibration, labelled stable isotope mixtures of each targeted amino acid as internal standard (IS) with exception of isoleucine and histidine (leucine IS and 3-Methylhistidine were assigned respectively), mobile phases, reagents, chromatographic and mass detection parameters of the method with modified sample preparation procedure comprising an acidic hydrolysis process. The concentrations of the targeted amino acids were determined using both electrospray ionization (ESI) and multiple reactions monitoring (MRM). In this section we specified general process, detailed settings information are in the related forthcoming paragraph with Table 1a and b. The samples were hydrolyzed as follows: 0.5 g of sample was hydrolyzed with 4 ml of acidic hydrolysis reagent in a screw capped glass tube for 24 h at 110ºC.

When the sample was cooled to room temperature (~24ºC), the hydrolyzed sample was centrifuged (Hettich Universal 320 desktop air cooled centrifuge) at 4000 rpm (g force: 3756.48) for 5 min. (No loss of sample solid phase remains at the bottom and necessary part is supernatant) After that, 10 µl of supernatant was transferred into a sample vial and completed to 1 ml with distilled water in order to obtain 800 fold diluted hydrolysates. Subsequent to the hydrolysis step, kit sample preparation procedures for calibration standards and samples were as follows: 50 µL of the standard or diluted hydrolysate was transferred into a sample vial. Next, 50 µL of the labeled stable isotopes mixture was added as an internal standard and 700 µL of reagent-1 were added to the sample vial before swirling for 5 sec.

HPLC system was operated to inject 3 µL of prepared sample into the Jasem analytical column specified for amino acid analysis (depending on the analysis kit) maintained at 30ºC. Chromatographic separation was carried out using Jasem's mobile phase A and B with gradient elution at a flow rate of 0.7 ml/min. The HPLC elution was as follows: the initial

LC gradient of 22% A was held for 1 min. Then, the gradient was ramped to 78% A in 3 min. and held for 0.5 min. Finally, the column was equilibrated at 22% A for 3 min. The total running time was 7.5 min. Mass spectrometric detection was performed on Agilent 6460 triple quadrupole MS equipped with an ESI source in the positive ion mode. The optimal MS detector settings were as follows: drying gas temperature 150°C, drying gas flow 10 L/min, nebulizer pressure 40 psi (Gauge-Nebulizing takes place in a chamber in which is under the atmospheric pressure not in the vacuum) and capillary voltage of 2000 V (+). The positive ESI mode was operated for the detection of amino acid and IS as protonated form ($m/z = [M+1]^+$). Collision-induced dissociation (CID) of this precursor ion produced one major product ion for each amino acid and IS. MRM transitions of the amino acid and corresponding IS (precursor ion to product ion) were monitored at optimum fragmentation voltages (FV) and optimum collision energies (CE) (Table 1a and 1b). The peak area ratio of the amino acid to the assigned IS was evaluated for quantification of targeted amino acid concentration.

Table 1a. MRM transitions of amino acids and conditions

Compound Name	Precursor Ion (m/z)	Product Ion (m/z)	FV (v)	CE (v)
Phenylalanine	166.1	120.1	80	6
Tyrosine	182.1	165	80	1
Methionine	150.1	104.1	80	4
Aspartic acid	134.1	74.1	90	10
Threonine	120.2	74.2	80	4
Serine	106.2	60.2	80	4
Alanine	90.2	44.2	80	4
Glycine	76.2	30.1	80	1
Proline	116.2	70.2	90	12
Cystine	241.1	74.2	100	24
Arginine	175.2	70.2	110	20
Histidine	156.1	110.1	100	8
Ornithine	133.2	70.3	80	14
Lysine	147.1	84.2	80	12
Glutamic acid	148.1	84.2	80	12
Leucine	132.2	43.3	100	24
Isoleucine	132.2	69.2	100	14
Valine	118.2	72.2	80	4

Table 1b. MRM transitions of amino acids and conditions

Compound Name	Precursor Ion (m/z)	Product Ion (m/z)	FV (v)	CE (v)
Phenylalanine IS	175.1	129.1	100	8
Tyrosine IS	192.1	145.2	80	8
Methionine IS	153.1	107.2	80	6
Aspartic acid IS	137.1	91.2	90	5
Threonine IS	121.1	75	80	6
Serine IS	109.1	63	90	8
Alanine IS	94.1	48.2	90	6
Glycine IS	78.2	31.3	90	4
Proline IS	122.1	75.2	90	14
Cystine IS	244.9	153.9	90	8
Arginine IS	177.2	70.2	110	20
3-Methyl histidine IS	173.2	127.2	80	10
Ornithine IS	138.2	74.2	80	16
Lysine IS	151.1	88.1	90	16
Glutamic acid IS	150.1	85.2	80	12
Leucine IS	142.2	96.3	120	6
Valine IS	126.1	80.2	80	8

Statistical Analysis

One-way ANOVA was used to determine the amino acids difference in different tissues of whiting. The statistical analyses were done using the software package PAST version 1.94b (Hammer *et al.*, 2001). Averages of significant variance sources were compared using Tukey's pair-wise comparisons test at a statistical significant level of 0.05.

Results

A total of 19 amino acids (AA) were detected in the different tissues of whiting samples (Table 2, Fig. 1). The essential amino acids (EAA) are Arg, Cys, His, Iso, Leu, Lys, Met, Phe, Thr, Tyr and Val. These EAA in different tissues of whiting constituted approximately 55.9% in meat, 54.8% in ovary and 51.7% in liver of total amino acids. The non-essential amino acids (NEAA) are Ala, Asp, Glu, Gly, Orn, Pro, Ser and Tau. The NEAA of whiting constituted about 44.1 per cent in meat, 45.2 per cent in ovary and 48.3 per cent in liver of total amino acids.

The EAA and the NEAA contents in different tissues of whiting are generally $AA_{Meat} \geq AA_{Ovary} > AA_{Liver}$ (Fig. 1). Namely, the NEAA; Asp, Glu, Ala and the EAA; Lys, Leu, Iso, Arg values were determined the higher values in meat > ovary > liver. Similar trends were obtained for other the EAA and the NEAA values (Fig. 1). It was found out that the most abundant the NEAA in meat, ovary and liver were Glu, Asp, Ala and Gly. Moreover, the most abundant the EAA in different tissues were determined as Lys, Leu, Iso, Arg and Val (Table 2). The amino acid contents except for Arg, Glu, Pro and Tau in meat and ovary of whiting determined statistically close to each other ($P > 0.05$), but the AA contents except for Cys, Tyr, Asp, Orn and Tau of these two tissues were determined statistically higher than AA contents of liver ($P < 0.05$) (Table 2).

Table 2. The mean values ± SE of amino acid in different tissues (meat, ovary and liver) on raw weight of Whiting (*Merlangus merlangus euxinus*) in the Black Sea

EAA/NEAA	Amino acids	Amino acid values in tissues (g/100 g)		
		Meat	Ovary	Liver
EAA	Arginine	1.29±0.010[a]	1.22±0.005[b]	0.44±0.005[c]
	Cystine	0.15±0.005[a]	0.14±0.030[a]	0.06±0.005[a]
	Histidine	0.00±0.000	0.28±0.280	0.00±0.000
	Isoleucine	0.97±0.135[a]	0.84±0.120[a]	0.28±0.030[b]
	Leucine	1.76±0.185[a]	1.74±0.145[a]	0.64±0.045[b]
	Lysine	2.25±0.270[a]	1.57±0.180[a]	0.55±0.095[b]
	Methionine	0.71±0.085[a]	0.61±0.070[a]	0.26±0.045[b]
	Phenylalanine	0.85±0.025[a]	0.92±0.025[a]	0.38±0.005[b]
	Threonine	0.88±0.040[a]	0.87±0.130[a]	0.27±0.065[b]
	Tyrosine	0.98±0.185[a]	0.72±0.135[a]	0.26±0.045[a]
	Valine	1.06±0.050[a]	1.05±0.020[a]	0.51±0.020[b]
Total (EAA)		11.695	10.89	3.915
NEAA	Alanine	1.34±0.030[a]	1.17±0.045[a]	0.46±0.010[b]
	Aspartic acid*	2.43±0.530[a]	2.11±0.375[a]	0.75±0.150[a]
	Glutamic acid*	3.34±0.075[a]	2.86±0.055[b]	1.08±0.020[c]
	Glycine	0.94±0.085[a]	1.15±0.040[a]	0.55±0.020[b]
	Ornitine	0.11±0.005[a]	0.12±0.000[a]	0.11±0.000[a]
	Proline	0.78±0.010[a]	1.06±0.010[b]	0.43±0.005[c]
	Serine	0.83±0.045[a]	0.95±0.050[a]	0.31±0.025[b]
	Taurine	0.29±0.020[a]	0.52±0.025[b]	0.29±0.005[a]
Total (NEAA)		9.215	8.970	3.660
Total (AA)		20.91	19.86	7.575

SE: standard error, AA: amino acid, EAA: essential amino acid, NEAA: non- essential amino acid. Values with different superscripts in same row are significantly different ($P < 0.05$). * Under the condition of acidic hydrolysis, glutamine and asparagine are entirely converted to glutamic acid and aspartic acid respectively

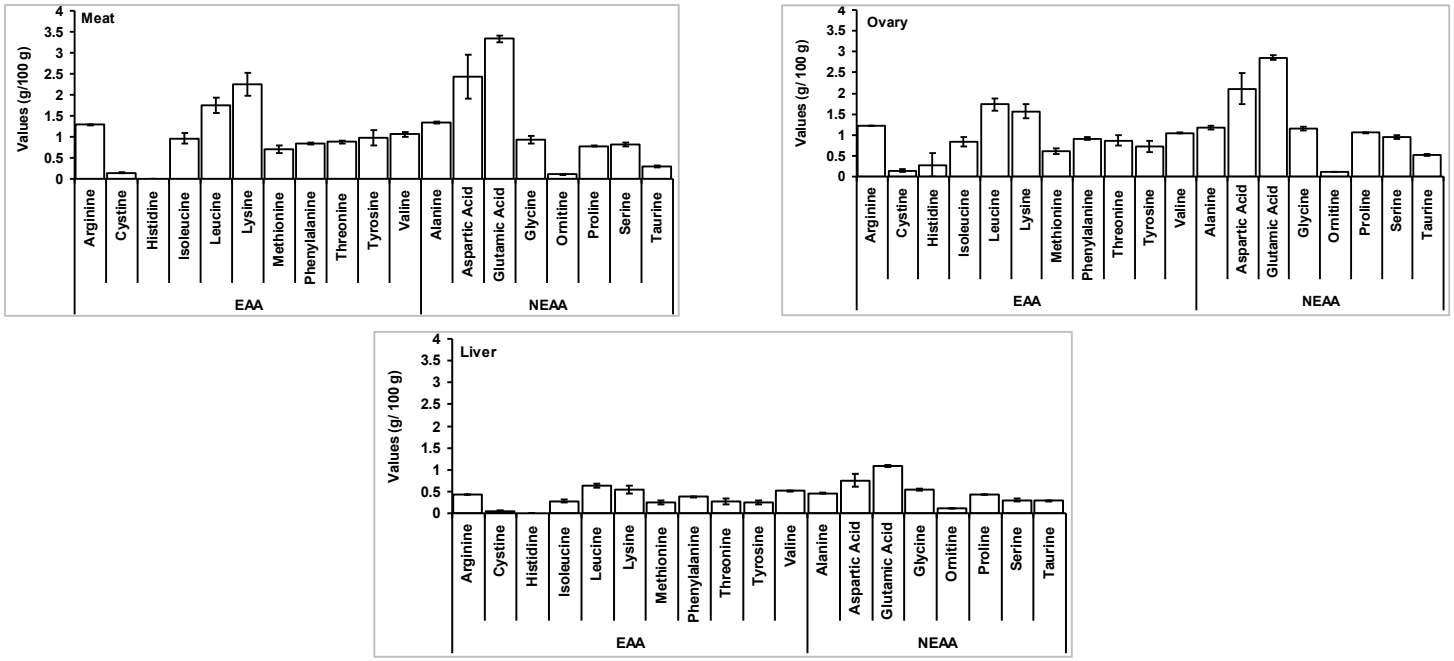

Figure 1. The amino acid composition in different tissues (meat, ovary and liver) on raw weight of whiting (*Merlangus merlangus euxinus*) in the Black Sea

Table 3. The five most abundant amino acids in muscle of different fish species from different geographical areas

Species	Origin	Five most abundant amino acids (AAs)	Ref.
Pleuronectes ferruginea	Aquaculture	Glutamic acid > aspartic acid > lysine > glycine > leucine	1
Hippoglossus hippoglossus	Aquaculture	Glutamic acid > aspartic acid > lysine > leucine > arginine	1
Paralichthys olivaceus	Aquaculture	Glutamic acid > aspartic acid > lysine > leucine > arginine	1
Pseudosciaena crocea	Xiamen aBay of China	Glutamic acid > aspartic acid > lysine > leucine > arginine	2
Lateolabrax japonicas	Xiamen Bay of China	Glutamic acid > aspartic acid > lysine > leucine > arginine	2
Pagrosomus major	Xiamen Bay of China	Glutamic acid > aspartic acid > lysine > leucine > arginine	2
Seriola dumerili	Xiamen Bay of China	Glutamic acid > aspartic acid > lysine > leucine > arginine	2
Hapalogenys nitens	Xiamen Bay of China	Glutamic acid > aspartic acid > leucine > lysine > arginine	2
Clarias anguillarias	Market in Ado Ekiti, Nigeria	Glutamic acid > aspartic acid > leucine > lysine > arginine	3
Oreochromis niloticus	Market in Ado Ekiti, Nigeria	Glutamic acid > aspartic acid > leucine > lysine > arginine	3
Cynoglossus senegalensis	Market in Ado Ekiti, Nigeria	Glutamic acid > aspartic acid > leucine > arginine > lysine	3
Trachurus trachurus	Market in Istanbul, Turkey	Glutamic acid > aspartic acid > lysine > leucine > valine	4
Zues faber	Aegean Sea	Glutamic acid > aspartic acid > lysine > leucine > alanine	5
Trigla lucerna	Marmara Sea	Glutamic acid > phenylalanine > aspartic acid > lysine > alanine	5
Scorpaena scrofa	Marmara Sea	Glutamic acid > lysine > aspartic acid > arginine > leucine	5
Scorpaena porcus	Marmara Sea	Proline > phenylalanine > glutamic acid > lysine > leucine	5
Merluccius merluccius	Marmara Sea	Proline > phenylalanine > glutamic acid > lysine > leucine	5
Lophius piscotorius	Marmara Sea	Proline > glutamic acid > phenylalanine > lysine > leucine	5
Trachinus draco	Marmara Sea	Proline > phenylalanine > glutamic acid > lysine > leucine	5
Esox lucius	Edirne Lake in Turkey	Proline > glutamic acid > phenylalanine > aspartic acid > lysine	5
Psetta maxima	Black Sea	Phenylalanine > glutamic acid > aspartic acid > lysine > leucine	5
Upeneus moluccensis	Antalya Gulf of Turkey	Lysine > leucine > aspartic acid > glutamic acid > alanine	6
Engraulis encrasicolus	Market in Istanbul, Turkey	Lysine > leucine > arginine > glutamic acid > aspartic acid	7
Pomatomus saltatrix	Market in Istanbul, Turkey	Lysine > leucine > arginine > glutamic acid > aspartic acid	7
Sarda sarda	Market in Istanbul, Turkey	Lysine > leucine > arginine > glutamic acid > aspartic acid	7
Murlus surmelutus	Market in Istanbul, Turkey	Lysine > leucine > arginine > glutamic acid > aspartic acid	7
Merlangius merlangus	Market in Istanbul, Turkey	Lysine > leucine > arginine > glutamic acid > aspartic acid	7

[1]: Kinm and Lall (2000); [2]: Limin *et al*. (2006); [3]: Adeyeye (2009); [4]: Erkan *et al*. (2010a); [5]: Özden and Erkan (2011); [6]: Doğan and Ertan (2017); [7]: Erkan *et al*. (2010b)

Discussion

Five the most abundant amino acids in muscle of different fish species from different geographical areas are shown in Table 3. In the previous studies Glu, Asp, Lys, Gly and Leu are determined as the most abundant five amino acids in muscle of different fish species such as *Pleuronectes ferruginea* (Storer, 1839), *Hippoglossus hippoglossus* (Linnaeus, 1758), *Paralichthys olivaceus* (Temminck & Schlegel, 1846), *Pseudosciaena crocea* (Richardson, 1846), *Lateolabrax japonicus* (Cuvier, 1828), *Pagrosomus major* (Temminck & Schlegel, 1843), *Seriola dumerili* (Risso, 1810), *Hapalogenys nitens* (Temminck & Schlegel, 1843), *Clarias anguillarias* (Linnaeus, 1758) and *Oreochromis niloticus* (Linnaeus, 1758) (Kinm and Lall, 2000; Limin *et al*., 2006; Adeyeye, 2009). Therefore, Özden and Erkan (2011) reported this classification as proline, Phe or Glu, Lys or Leu in different marine and inland water fish species such as *Scorpaena porcus* Linnaeus, 1758, *Merluccius merluccius* (Linnaeus, 1758), *Lophius piscotorius* Linnaeus, 1758, *Trachinus draco* Linnaeus, 1758 and *Esox lucius* Linnaeus, 1758 from different geographical areas. Erkan *et al*. (2010b) reported that Lys, Leu, Arg, Glu and Asp are the most abundant amino acids in different raw marine fish species (*Engraulis encrasicolus* (Linnaeus, 1758), *Pomatomus saltatrix* (Linnaeus, 1766), *Sarda sarda* (Bloch, 1793), *Mullus surmelutus* Linnaeus, 1758 and *Merlangius merlangus* (Linnaeus, 1758)). Moreover, Phe and Lys reported as the most abundant AAs for *Psetta maxima* (Linnaeus, 1758) in the Black Sea (Özden and Erkan, 2011) and *Upeneus moluccensis* (Bleeker, 1855) in the Mediterranean (Doğan and Ertan, 2017).

We did not detect any study about AAs contents in different tissues of whiting. For that reason, we create Table 3 with different fish species and we found generally similar results for the most abundant amino acid contents (e.g. NEAA: Asp, Glu; EAA: Leu and Lys). Our data showed that the amino acid contents including Arg, Glu, Pro and Tau in meat and ovary of whiting was statistically different each other ($P < 0.05$), and also the AA contents except for Cys, Tyr, Asp, Orn and Tau of these two tissues were statistically higher than AA contents of liver ($P < 0.05$). In general, when looking at figure 2, it can be seen that the AAs contents in different tissues of whiting are $AA_{Meat} \geq AA_{Ovary} > AA_{Liver}$. These results showed that whiting ovary approximately have AAs content as much as meat AAs content.

The EAA of whiting constituted approximately 55.9% in meat, 54.8% in ovary and 51.7% in liver of total amino acids. The NEAA of whiting constituted about 44.1% in meat, 45.2% in ovary and 48.3% in liver of total amino acids. These ratios for the EAA values were reported between 42 - 57% for 9 fish species, 37 - 47% for 6 crustaceans and 34 - 56% for 6 mollusc species (Özden and Erkan, 2011).

The above values demonstrated that considerable variations in amino acid levels can be obtained from the different and/or same fish species. Such variations are possibly a result of several factors including differences in feeding,

season, species, sex, stage of maturity, nutritional, and environmental condition as well as methods used for AA determination (Kinm and Lall, 2000; Limin *et al*., 2006; Adeyeye, 2009; Erkan *et al*., 2010ab; Özden and Erkan, 2011; Doğan and Ertan, 2017). In the classical method (HPLC), the sample is derivatized while being made ready for analysis. However, there is no derivatization in the LC-MS/MS method and the sample is directly hydrolyzed. Also, with the LC-MS/MS method, it is possible to work with a much lower sample (e.g. 0.5 g) than the classical methods.

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