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RESEARCH ARTICLE ARAŞTIRMA MAKALESİ

Optimization of hydrolyzation procedure for amino acid analysis in fish meat with HPLC-DAD by Response Surface Methodology (RSM)

Balık etinde HPLC-DAD ile yapılan amino asit analizi hidrolizasyon prosedürünün Yanıt Yüzey Metodu (RSM) ile optimizasyonu

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Abstract: In this research, with the aim of maximizing amino acid content by different hydrolyzation procedures of fish meat were carried out and analysed with high performance liquid chromatography (HPLC) equipped with diode array detector (DAD). Response surface methodology (RSM) was used to determine factors that used in the experiments. The RSM suggested 16 different hydrolyzation trials between the ranges of normality as 3 N - 8 N, temperature as 90°C-110°C and duration as 12-24 hours were run. In the following, hydrolysed samples were analysed with HPLC-DAD and obtained data were evaluated with Box-Behnken method in RSM. Our results show that, the most effective experiment was found as hydrolysing by 3.79 N at 110°C in 24 hours for total amino acid content as well as maximized essential amino acids with 0.981 desirability. For sensitive ones, hydrolysing in 3.42 N at 106.8°C in 12.02 hours was found as most effective with maximized Asn, Gln and Trp with 0.849 desirability.

Keywords: Amino acid, Box-Behnken, seafood, pre-treatment, response surface methodology, hydrolyzation

Öz: Bu çalışmada; diyot dedektörü (DAD) donanımlı yüksek performanslı likit kromatografi (HPLC) ile balık etinde gerçekleştirilen amino asit analizinde, amino asit miktarını maksimize etmek için farklı hidrolizasyon prosedürleri gerçekleştirilmiştir. Deneylerde kullanılan faktörleri belirlemek için yanıt yüzey metodu (RSM) kullanılmıştır 16 adet farklı hidrolizasyon deneyi RSM ile kurulmuş olup, 3N-8N arasındaki normalite değerleri, 90°C-110°C arasındaki sıcaklık değerleri ve 12-24 saat arasındaki süre değerleri dikkate alınmıştır. Elde edilen hidrolizatlar HPLC-DAD ile analiz edilmiş olup elde edilen veri RSM'deki Box-Behnken metoduna göre değerlendirilmiştir. Sonuçlara göre; toplam ve esansiyel amino asitler için en efektif hidrolizasyon metodu 3,29 normalitede, 110°C sıcaklıkta, 24 saat olarak belirlenmiş olup istenilebilirlik değeri 0,981 olarak tespit edilmiştir. Hassas amino asitler için ise; 3,42 normalitede, 106,8°C sıcaklıkta, 12,02 saat süre Asn, Gln ve Trp için maksimum değerleri vermiş olup istenilebilirlik değeri 0,849 olarak tespit edilmiştir.

Anahtar kelimeler: Amino asit, Box-Behnken, su ürünleri, ön işlem, yanıt yüzey metodu, hidrolizasyon

INTRODUCTION

Fish and other seafood are ideal food sources for a healthy diet (Hosomi et al., 2012) with high composition of omega 3 fatty acids (Musarskaya et al., 2018), some beneficial nutrients (Biesalski, 2005) low carbohydrate content (Krzynowek & Murphy, 1987) and high quality proteins (Tahergorabi & Hosseini, 2018). That protein content has biologically important (Köksal & Özel, 2008) and well-balanced amino acid composition (Lund, 2013) as well as rich in terms of essential amino acids (Çankırılıgil & Berik, 2017) which are fundamental for humans (Hou et al., 2015). Essential amino acids are plays such roles in metabolism just as protein synthesis, hormonal secretions, gene expression and cell signalling (Wu, 2009). Besides, non-essentials also contribute to body growth and health (Wu et al., 2013) along with essentials ones.

Considering these benefits, several studies that carried out on amino acid composition of various fish species. Thus, determination techniques of amino acids had gained importance parallel with such studies with the aim of getting more concise results. Despite the fact that, developing several well-designed analyse methods as rapid and accurate, these methods can be as good as the applied hydrolyzation procedure of samples scarcely due to severe conditions of hydrolyzation that caused degradation of amino acid content. Conventional hydrolyzation procedures are based on the digesting of fish meat with hydrochloric acid having high normality at high temperature for hours in drying oven. However, these factors affects each amino acids with different ways and some of them can be degraded and should be well-optimized.

The optimization of experimental design is very important when the analytical procedures are developed and validated (Vera et al., 2014). Response surface methodology (RSM) as an optimization tool for analytical methods (Bezerra et al., 2008) and using for optimization of all chemical and biochemical processes (Baş & Boyacı, 2007). RSM is using an empirical model building with considering relationships between independent variables and data that obtained by mathematical and statistical techniques from conducted experiments (Alvarez, 2000; Said Mohamad & Mohamed Amin, 2015) which is much more time saving than conventional designing methods (Widyaningsih et al., 2018). In this research, RSM was used to specify optimum conditions for pre-treatment of amino acid analysis. Before the applying RSM, the experimental design should be specified by choosing the factors that directly affect to responses (Hwang et al., 2016).

The aim of this study is to maximize essential amino acids and reducing losses of non-essential amino acids with applying different ranges of normality, temperature and duration times in the hydrolyzation procedure of fish meat. Furthermore, the composition of some vulnerable amino acids such as tryptophan (Trp), glutamine (Gln), asparagine (Asn) are decrease more than others when they exposed to high temperature and low pH (Varlık et al., 2004) whereas causing an increase on aspartic acid and glutamic acid (Dong et al., 2005), inversely. Also, the optimum conditions for aforementioned amino acids were determined. Ultimately, two suggested hydrolyzation conditions by the RSM were performed as pre-treatment for both essential amino acids and vulnerable ones.

MATERIAL AND METHODS

Chemical, solutions and consumables

Hydrochloric acid fuming %37 (HCl), sodium phosphate dibasic solution (Na_2HPO_4), sodium hydroxide (NaOH), methanol (MeOH; GC grade) and acetonitrile (ACN; GC grade) were purchased from Merck. Borate buffer, o-phthalaldehyde reagent (OPA), 9-fluorenylmethyl chloroformate reagent (FMOC), amino acid standard solutions (mix of L-alanine, L-arginine, L-aspartic acid, L-cystine, L-glutamic acid, glycine, L-histidine hydrochloride monohydrate, L-isoleucine, L-leucine, L-lysine hydrochloride, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, L-valine stored in 0.1N HCl with different dosages as 1nmol, 250pmol, 100pmol, 25pmol and 10pmol) and extended amino acid standards for sensitive ones such as L-glutamine, L-asparagine, L-tryptophan and L-4-hydroxyprolin in the form of powder were obtained from Agilent Technologies. Water purified (18.1 megaohm purity) with Thermo Scientific Nanopure. Amino acid column for HPLC system (Zorbax clip AAA, 5µm, 4.6x150m), 1.5ml amber vials with politetrafloroetilen caps (PTFE) and syringe filters (0.45µm) were also purchased from Agilent Technologies.

Fish material

In this research, rainbow trout (*Oncorhynchus mykiss*) was preferred as fish material for the purpose of the amino acid studies carried out with this species are very common and discussion were done considering such studies. Trouts were obtained from Central Fisheries Research Institute operated in Trabzon, Turkey and 50 individuals which are 250 g each were used. Firstly, trouts were washed and deskinned. Fillets were homogenized with Stomacher 400 Circulator in 300 rpm and samples were stored in +2°C for further stages.

Experimental design

In this study, the effects of three independent variables such as normality, temperature and time on amino acid content of fish meat were evaluated. Thus, the Box-Behnken analysis of response surface methodology (RSM) was used for experimental design. In this method, the most effective values of factors affecting responses were determined for selected responses. The maximum and minimum values of these factors were determined according to preliminary results and upper or lower values of these factors caused some false and irregularities in amino acid analysis due to excessive or insufficient hydrolysing. The values were coded as -1, 0 and +1 for minimum values, mean values and maximum values, respectively (Table 1). In the values ranging 1 N- 3 N, 70°C-90°C and 8-12 hours samples were not completely digested. Above the 8 N, 110°C and 24 hours caused losses of some amino acids completely. For this reason, the factors were selected as normality ranging from 3 N to 8 N, temperature ranging from 90°C to 110°C and duration ranging from 12 to 24 hours. Besides, content of total amino acids (TAA), total essential amino acids (TEAA), threonine (Thr), valine (Val), methionine (Met), phenylalanine (Phe), isoleucine (Iso), leucine (Leu), lysine (Lys), tryptophan (Trp), asparagine (Asn), aspartic acid (Asp), glutamine (Gln), glutamic acid (Glu), serine (Ser), glycine (Gly), histidine (His), alanine (Ala), tyrosine (Tyr), cysteine (Cys), hydroxyproline (Hyp) were selected as responses individually. Thus, according to Box-Behnken design, 16 experiments having differences in the combination of these factors were determined. In the following, 1 gram of homogenised fish meat and 10ml HCl which have different normalities put on autoclave bottle enduring high temperature and pressure were digested in drying oven in varied temperatures and duration time in accordance with design matrix. Box-Behnken design matrix of the dependent variables was shown in Table 1. All experiments were replicated 3 times (n=3).

Table 1. Box-Behnken design matrix of pre-treatment of fish meat for amino acid analyses

Design Order	Temperature (°C) X^1	Normality (N) X^2	Duration (hour) X^3	Responses Y
1	-1	-1	0	-
2	1	-1	0	-
3	-1	1	0	-
4	1	1	0	-
5	-1	0	-1	-
6	1	0	-1	-
7	-1	0	1	-
8	1	0	1	-
9	0	-1	-1	-
10	0	1	-1	-
11	0	-1	1	-
12	0	1	1	-
13	0	0	0	-
14	0	0	0	-
15	-1	-1	-1	-
16	1	1	1	-

*TAA, TEAA, Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Hyp, Ile, Leu, Lys, Met, Phe, Ser, Thr, Trp, Tyr, Val were selected as responses, individually.

Amino acid analyses

Subsequent to hydrolysing procedure, obtained samples were filtered by syringe filter (0.45µm), diluted as 10^{-1} with purified water and finally they stored in 1.5ml amber vials equipped with PTFE caps for the amino acid analysis. Amino acid analyses were carried out with Agilent HPLC Infinity II system equipped with diode array detector (DAD) and auto-sampler. Amino acid analyses were done according to the method of Henderson et al. (2000). In this method, 0.5µL sample was drawn from vial with an automatic sampler and it was derivatized with borate buffer, OPA and FMOC reagents. Subsequent to derivatization, samples were injected to the system individually. Separation of amino acids was carried out by Zorbax Eclipse AAA amino acid column at 40°C in gradual mobile phase which are mix of 40mM Na_2HPO_4 (A) adjusted to 7.8 pH with 10N NaOH and MeOH:ACN:H_2O (B) in the ratio of 45%:45%:10% with 2mL/min flow rate, approximately for 26 minutes. Gradient stages of mobile phase were applied as A:100%, B:0%; A:43%, B:57%; A:0%, B:100%; A:0%, B:100%; A:100%, B:0% in 1.9min, 18.1min, 18.6min, 22.3min, 23.2min, respectively. Detection was done in two different wavelengths in 338nm, 10nm bandwidth for OPA-amino acids and 262nm, 16nm bandwidth for FMOC-amino acids. All samples were analysed for 3 times (n=3). Obtained results were calibrated automatically and expressed as g/100g.

Data evaluation

Obtained results from amino acid analysis were written in response column in Box-Behnken design matrix and evaluated with Design Expert 7.16 software. All models were formulated in compliance with results along with the significance of models and lack of fit which were evaluated for all responses as TAA, TEAA, Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Hyp, Ile, Leu, Lys, Met, Phe, Ser, Thr, Trp, Tyr and Val. Besides, along with responses of TAA and TEAA, they were also evaluated with the sum of mentioned amino acids. Finally, optimum values for to get maximal total amino acid content as well as essential amino acids and maximized vulnerable amino acids were determined considering the results of 16 experiments. The used functions of models were given below.

$$y = \beta_0 + \sum \beta_i X_i \quad \text{(Linear model)}$$

$$y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j \quad \text{(Two factor interaction model)}$$

$$y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \quad \text{(Quadratic model)}$$

Where y is one of the responses, β_0 is the regression intercept, X symbols are independent variables, β_i is coefficient of the linear parameters, β_{ij} is coefficient of interaction between factors, and β_{ii} is the coefficient of the quadratic parameters (Davarnejad et al., 2018; Nadarajan et al., 2018).

Differences among amino acid composition of all experiments were determined by one-way analysis of variance one-way ANOVA in SPSS 12 after testing the homogeneity of the data (Levene test). Results were expressed as mean ±SD (n=9) and significance level was set as P≤0.05 (Zar, 1999).

Achieved optimum values of factors for both total amino acids and vulnerable ones were expressed as optimization 1 (O_1) and optimization 2 (O_2), respectively. In the evaluation of O_1, the essential amino acids except tryptophan were selected as for getting maximum along with other amino acids. In the second one (O_2), an additional optimization procedure was carried out for getting maximized some special amino acids that show variations in quantity with high temperature and low pH such as Trp, Asn, Gln. With these extreme conditions, Asn and Gln can be transformed to Asp and Glu, respectively. Therefore; Asp, Asn, Gln and Glu were evaluated separately along with Trp which is amongst most vulnerable ones. Ultimately, these two optimization procedures were applied as 17th and 18th experiments and amino acid composition of these experiments was determined and compared to previous runs.

RESULTS AND DISCUSSION

According to experiments, linear model was applied for TAA, TEAA, Glu, Ser, Gly, Thr, Tyr, Val, Met, Trp, Iso, Leu; two factor interaction (2FI) model was applied for Asp, Asn, Gln,

His, Ala, Lys; and quadratic model was applied Cys and Hyp. The final equations of coded factors for models are;

$$TAA = 11.76 + 3.14A - 0.66B + 0.49C$$
$$TEAA = 4.52 + 1.43A - 0.2B + 0.29C$$
$$Asp = 1.03 + 0.34A - 0.4B + 0.036C - 0.29AB - 0.045AC + 0.21BC$$
$$Glu = 1.64 + 0.47A - 0.14B + 0.1C$$
$$Asn = 0.46 + 0.14A - 0.064B + 0.016C - 0.092AB - 0.039AC + 0.049BC$$
$$Ser = 1.08 + 0.19A + 0.013B + 0.014C$$
$$Gln = 0.44 + 0.21A - 0.065B + 0.040C - 0.12AB - 0.054AC + 0.048BC$$
$$His = 0.38 + 0.03A - 0.06B + 0.009C - 0.033AB + 0.042AC + 0.042BC$$
$$Gly = 0.42 + 0.11A + 0.012B + 0.026C$$
$$Thr = 1.14 + 0.27A - 0.09B + 0.033C$$
$$Ala = 0.19 + 0.071A - 0.024B + 0.012C - 0.043AB - 0.019AC + 0.021BC$$
$$Tyr = 0.34 + 0.013A + 0.12B + 0.041C$$
$$Cys = 0.082 - 0.0064A - 0.0006B + 0.005C - 0.0005AB + 0.011AC + 0.015BC - 0.013A^2 - 0.018B^2 - 0.0052C^2$$
$$Val = 0.46 + 0.2A + 0.0011B + 0.063C$$
$$Met = 0.41 + 0.15A - 0.014B + 0.031C$$
$$Trp = 0.28 - 0.045A - 0.05B - 0.056C$$
$$Phe = 0.51 + 0.19A - 0.0032B + 0.05C$$
$$Iso = 0.35 + 0.19A + 0.0036B + 0.068C$$
$$Leu = 0.93 + 0.32A - 0.039B + 0.055C$$
$$Lys = 0.084 + 0.33A - 0.16B + 0.031C - 0.18AB - 0.097AC + 0.092BC$$
$$Hyp = 0.41 - 0.012A + 0.06B + 0.021C + 0.06AB + 0.026BC - 0.13A^2 - 0.039B^2 - 0.068C^2$$

Among these models, linear models of TAA and TEAA were found significant with a very low pure error. Analysis of variance for the responses of total amino acids and essential amino acids were shown in Table 2. The response surface models of amino acids and essential amino acids were also shown in Figure 1.

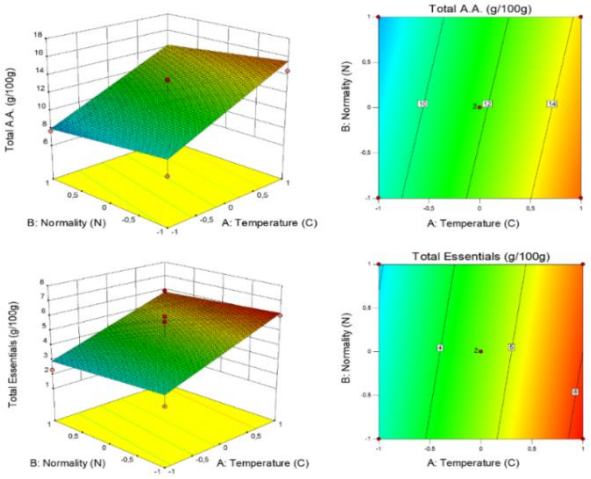

Figure 1. Response surface plots for total amino acids and essential amino acids

Table 2. Analysis of variance for models of total amino acids and essential amino acids

Total A.A.	**SS**	**df**	**MS**	**F Value**	**p-value**	
Linear Model	115.02	3	38.34	5.03	0.0143	significant
A:Temperature	112.54	1	112.54	14.76	0.0018	
B:Normality	4.87	1	4.87	0.64	0.4377	
C:Time	2.79	1	2.79	0.37	0.5552	
Residual	106.75	14	7.63			
Lack of Fit	106.75	13	8.21	2830.61	0.0147	significant
Pure Error	0.0029	1	0.00			
Total E.A.A.	**SS**	**df**	**MS**	**F Value**	**p-value**	
Linear Model	24.38	3	8.13	5.59	0.0099	significant
A:Temperature	23.42	1	23.42	16.1	0.0013	
B:Normality	0.44	1	0.44	0.3	0.5906	
C:Time	0.95	1	0.95	0.65	0.4329	
Residual	20.37	14	1.45			
Lack of Fit	20.37	13	1.57	7340.16	0.0091	significant
Pure Error	0.0002	1	0.0002			

*SS: Sum of squares, df: Degrees of freedom, MS: Mean squares.
**Tryptophan was evaluated separately apart from the essential amino acids due to sensitive high temperature and low pH. (A.A.: Amino acids, E.A.A.: Essential amino acids).

When the essential amino acids evaluated individually, all models except tryptophan were found significant. The linear model of methionine was determined as significant, in spite of the model's lack of fit was found as not significant. Analysis of variance for the responses of some amino acids were shown in Table 3.

Table 3. Analysis of variance for some models of amino acids

Response	SS	df	MS	F Value	p-value	
THR (Linear model)	0.86	3	0.29	3.41	0.0474	significant
Lack of Fit	1.17	13	0.090	296.10	0.0455	significant
VAL (Linear model)	0.51	3	0.17	7.99	0.0024	significant
Lack of Fit	0.30	13	0.023	348.25	0.0419	significant
MET (Linear model)	0.28	3	0.095	6.37	0.0060	significant
Lack of Fit	0.21	13	0.016	100.82	0.0778	not significant
PHE (Linear model)	0.45	3	0.15	5.62	0.0097	significant
Lack of Fit	0.37	13	0.029	306.86	0.0447	significant
ISO (Linear model)	0.51	3	0.17	9.46	0.0011	significant
Lack of Fit	0.25	13	0.019	714.55	0.0293	significant
LEU (Linear model)	1.20	3	0.40	4.96	0.0150	significant
Lack of Fit	1.13	13	0.087	493.23	0.0352	significant
LYS (2FI model)	1.52	6	0.25	6.40	0.0042	significant
Lack of Fit	0.43	10	0.043	4074.21	0.0122	significant
ASP (2FI model)	2.91	6	0.49	6.58	0.0037	significant
Lack of Fit	0.81	10	0.081	25311.75	0.0049	significant
GLU (Linear model)	4.20	3	1.40	4.56	0.0198	significant
Lack of Fit	4.29	13	0.33	32.86	0.1358	not significant

* SS: Sum of squares, df: Degrees of freedom, MS: Mean squares

The most appropriate model was selected as 2FI model via Box-Behnken design matrix in Design Expert 7.16 software for asparagine and glutamine. According to models, the lack of fit parameters were found not significant instead of models found as significant same as glutamic acid (Table 3). As we mentioned before, asparagine and glutamine are sensitive to high temperature and they can transform to aspartic acid and glutamic acid in the presence of heat and low pH (Dong et al., 2005).

However; Asp, Glu, Asn and Gln are increasing together with the heat and pH in spite of this transforming process. Our results show that, asparagine and glutamine are increasing till the some point due to degradation of proteins to amino acids by digestion process. That's the reason why the models have shown similarities for independent variables contrary to expectations. Analysis of variance for the responses of asparagine and glutamine shown in Table 4. The response surface models of Gln and Asn were shown in Figure 2.

Table 4. Analyses of variance for models of asparagine and glutamine

ASN	SS	df	MS	F Value	p-value	
2FI Model	0.28	6	0.047	4.66	0.0135	significant
A:Temperature	0.2	1	0.2	20.17	0.0009	
B:Normality	0.042	1	0.042	4.17	0.0658	
C:Time	0.0026	1	0.0026	0.26	0.6185	
AB	0.058	1	0.058	5.75	0.0354	
AC	0.0094	1	0.0094	0.94	0.3542	
BC	0.016	1	0.016	1.63	0.2276	
Residual	0.11	11	0.01			
Lack of Fit	0.11	10	0.011	191.02	0.0563	not significant
Pure Error	0.0001	1	0.0001			
GLN	**SS**	**df**	**MS**	**F Value**	**p-value**	
2FI Model	0.59	6	0.099	6.29	0.0045	significant
A:Temperature	0.46	1	0.46	29.25	0.0002	
B:Normality	0.044	1	0.044	2.8	0.1224	
C:Time	0.017	1	0.017	1.11	0.3154	
AB	0.099	1	0.099	6.27	0.0293	
AC	0.019	1	0.019	1.18	0.3012	
BC	0.016	1	0.016	0.99	0.3414	
Residual	0.17	11	0.016			
Lack of Fit	0.17	10	0.017	33.74	0.1333	not significant
Pure Error	0.0005	1	0.0005			

* SS: Sum of squares, df: Degrees of freedom, MS: Mean squares

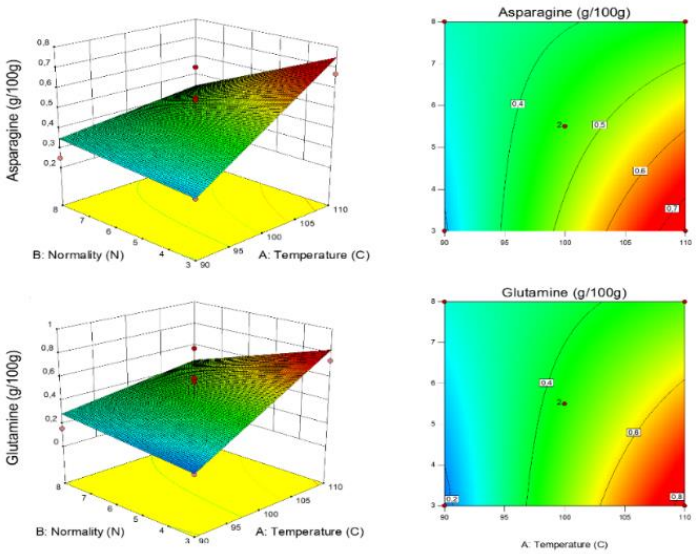

Figure 2. Response surface plots for asparagine and glutamine

Tryptophan is vulnerable for high temperatures and low pH (Marinho et al., 2015; Varlık et al., 2004) and the most intense losses occur at 100°C in the pH ranging 2 to 7 (Cuq & Firedman, 1989). Thus, it can be easily degraded during any pre-treatment based on digestion with the acidic environment at high temperatures. So, with the aim of more concise results, tryptophan was evaluated separately from the others due to this unique structure.

Linear model was applied for tryptophan which is determined via Box-Behnken design matrix. According to results, neither model nor lack of fit parameters found significant. According to our results, its severe conditions that directly changes the composition of tryptophan have an effect on the model. Analysis of variance for the response and surface response plot of tryptophan were shown in Table 5 and Figure 3.

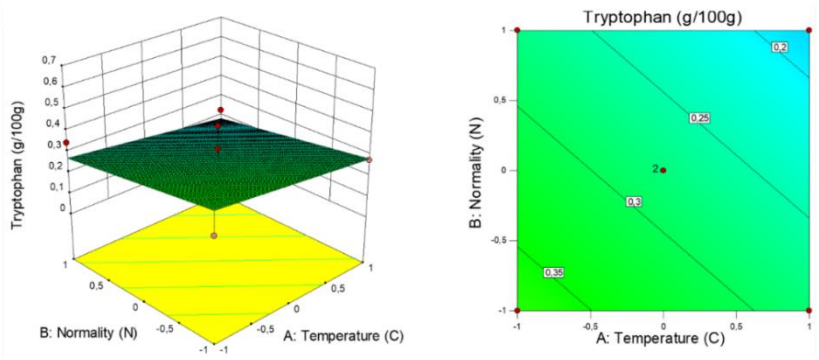

Figure 3. Response surface plot of tryptophan

Table 5. Analysis of variance for the model of tryptophan

TRP	SS	df	MS	F Value	p-value	
Linear Model	0.11	3	0.037	1.32	0.3062	not significant
A:Temperature	0.023	1	0.023	0.82	0.3818	
B:Normality	0.028	1	0.028	1	0.3341	
C:Time	0.036	1	0.036	1.28	0.2768	
Residual	0.4	14	0.028			
Lack of Fit	0.39	13	0.03	5.29	0.3292	not significant
Pure Error	0.0057	1	0.0057			

* SS: Sum of squares, df: Degrees of freedom, MS: Mean squares

The obtained results were evaluated with numerical optimization option in Design Expert 7.16 software. In numerical optimization, responses of all amino acids which is amino acid content (y) in our study can be selected as "maximum", "minimum", "in the range" and "exclude". With this selection, the amounts of amino acids can be maximized or minimized with regard to factors (X^1, X^2 and X^3) which are values of normality, temperature and duration in this study. In optimization one (O_1), essential amino acids except tryptophan were selected as "maximum" and the rest of them selected as "in the range" choice with the aim of getting maximum of essential amino acids along with moderate non-essential amino acids. According to results, the most reliable solution having the highest desirability (0.981) specified as hydrolysing in 3.79N at 110°C in 23.98 hours. Desirability ramp for optimization procedure 1 shown in Figure 4.

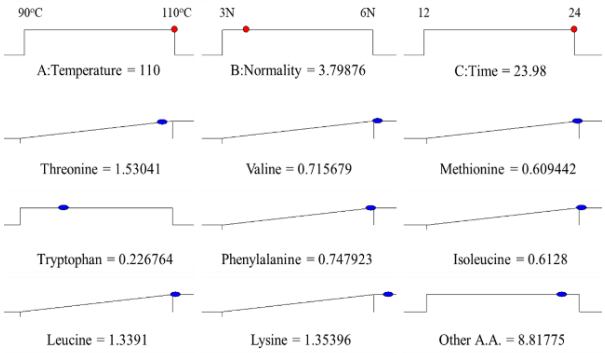

Figure 4. Optimized hydrolyzation procedure for essential amino acids. All essential amino acids except of tryptophan were selected as "maximum", the sum of remain amino acids and tryptophan selected as "in the range"

In optimization two (O_2), tryptophan, asparagine and glutamine were selected as "maximum'' and aspartic acid and glutamic acid selected as "in the range" choice with the aim of getting maximum of sensitive ones along with moderate Asp-Glu. According to results, the most convenient solution having the highest desirability (0.849) specified as hydrolysing in 3.42 N at 106.8°C in 12.02 hours. Desirability ramp for optimization procedure 2 shown in Figure 5.

After the optimization, achieved two optimizations procedure were applied and compared to previous runs. According to results; glutamine, serine, histidine, threonine, tyrosine, valine, phenylalanine, ısoleucine, leucine, lysine and total amino acids were found highest statistically in the optimization 1 (O_1) among all experiments.

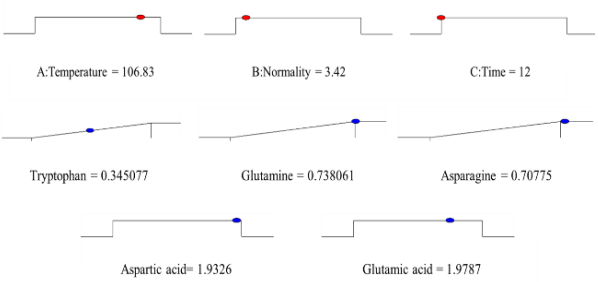

Figure 5. Optimized digestion procedure for sensitive amino acids. While tryptophan, asparagine and glutamine were selected as maximum, aspartic acid and glutamic acid were selected as in the range

Only methionine was detected as the second highest in O_1 consonant with their model. Considering the importance of the essential amino acids along with non-essentials which are supports growth and maintaining health (Hou et al., 2015; Lund, 2013; Wu, 2009; Wu et al., 2013), the hydrolyzation procedure 1 (O_1) can be suggested as a pre-treatment for amino acid analyses in the fish meat.

Asparagine and glutamine are vulnerable to high temperature and they can transform to aspartic and glutamic acids by high temperature. Several researchers are either not analysed asparagine and glutamine (Chen et al., 2007; Kang et al., 2014; Mohanty et al., 2014; Unusan, 2007) or they stated that asparagine and glutamine are very low or none in the fish meat (Boonyoung et al., 2012; Dong et al., 2005; Rebolé et al., 2015; Sarma et al., 2013) contrary to aspartic acid and glutamic acid which are found highest amino acids, often (Özden, 2005; Park et al., 2006). Reason for this, asparagine and glutamine are directly affected by the severe conditions of the digestion process. In this research, after the optimization 2 (O_2), the highest asparagine and glutamine were detected with moderate aspartic acid and glutamic acid.

Tryptophan is also not detected in most of the studies related to various fish species (Boonyoung et al., 2012; Galla et al., 2012; Joshi et al., 2017; Oluwaniyi et al., 2010). Despite the fact that the tryptophan model is not significant, the second pre-treatment suggestion (O_2) is more successful than other experiments. In this research, one of the main aims is to determine the rapid and precise digestion procedure for amino acid analysis aforementioned before. For this reason, optimization 2 can be used for total determination of sensitive amino acids just like tryptophan, asparagine, glutamine along with aspartic acid and glutamic acid.

For more concise and precise results for tryptophan individually, other digestion procedures based on alkaline digestion methods (Bech-Andersen, 1991; Nielsen & Hurrell, 1984) or enzymatic hydrolysis (Kurozawa et al., 2008; Nilsang et al., 2005) can be used. The obtained amino acid results of the experiments were shown in Table 6.

Table 6. Amino acid composition of fish meat according to conducted experiments (g/100g)

D.O.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	O_1	O_2
ASP	1.04^{e}	1.93^{a}	0.35^{h}	0.98^{e}	0.63^{g}	1.15^{e}	0.79^{f}	1.19^{e}	1.79^{b}	0.41^{h}	1.66^{c}	0.73^{f}	1.14^{e}	1.14^{e}	0.74^{f}	0.81^{f}	1.41^{d}	1.02^{e}
(±SE)	*±0.06*	*±0.06*	*±0.08*	*±0.04*	*±0.02*	*±0.05*	*±0.03*	*±0.05*	*±0.17*	*±0.05*	*±0.08*	*±0.04*	*±0.13*	*±0.05*	*±0.06*	*±0.08*	*±0.06*	*±0.05*
GLU	1.04^{f}	2.54^{a}	0.68^{g}	2.01^{c}	1.08^{f}	2.18^{b}	1.18^{f}	2.24^{ab}	2.51^{a}	0.92^{fg}	2.65^{a}	1.07^{f}	2.02^{c}	2.16^{b}	0.79^{fg}	1.76^{d}	2.53^{a}	1.51^{e}
(±SE)	*±0.03*	*±0.22*	*±0.02*	*±0.06*	*±0.05*	*±0.04*	*±0.06*	*±0.10*	*±0.26*	*±0.12*	*±0.15*	*±0.11*	*±0.22*	*±0.06*	*±0.06*	*±0.13*	*±0.04*	*±0.04*
ASN	0.29^{f}	0.67^{ab}	0.25^{f}	0.54^{c}	0.35^{e}	0.56^{c}	0.38^{e}	0.52^{c}	0.62^{b}	0.27^{f}	0.60^{b}	0.36^{e}	0.55^{c}	0.54^{c}	0.21^{g}	0.45^{d}	0.61^{b}	0.77^{a}
(±SE)	*±0.01*	*±0.03*	*±0.01*	*±0.02*	*±0.01*	*±0.01*	*±0.04*	*±0.03*	*±0.07*	*±0.03*	*±0.02*	*±0.05*	*±0.03*	*±0.02*	*±0.01*	*±0.01*	*±0.02*	*±0.02*
SER	0.84^{ef}	1.17^{cd}	0.79^{f}	1.35^{b}	0.87^{e}	1.24^{c}	0.70^{g}	1.12^{cd}	1.33^{b}	0.93^{e}	1.28^{c}	1.08^{d}	1.22^{c}	1.17^{cd}	0.71^{g}	1.23^{c}	1.64^{a}	1.12^{cd}
(±SE)	*±0.01*	*±0.02*	*±0.02*	*±0.21*	*±0.11*	*±0.13*	*±0.04*	*±0.19*	*±0.16*	*±0.01*	*±0.02*	*±0.02*	*±0.03*	*±0.02*	*±0.03*	*±0.14*	*±0.02*	*±0.02*
GLN	0.17^{h}	0.74^{b}	0.16^{h}	0.57^{d}	0.17^{h}	0.59^{d}	0.24^{g}	0.56^{d}	0.54^{d}	0.25^{g}	0.66^{c}	0.35^{f}	0.59^{d}	0.56^{d}	0.11^{i}	0.46^{e}	0.44^{e}	0.81^{a}
(±SE)	*±0.01*	*±0.02*	*±0.03*	*±0.02*	*±0.01*	*±0.01*	*±0.02*	*±0.03*	*±0.06*	*±0.03*	*±0.01*	*±0.06*	*±0.05*	*±0.00*	*±0.01*	*±0.02*	*±0.01*	*±0.03*
HIS	0.41^{b}	0.49^{ab}	0.27^{d}	0.42^{b}	0.39^{c}	0.27^{d}	0.33^{cd}	0.43^{b}	0.56^{a}	0.29^{cd}	0.49^{ab}	0.33^{cd}	0.43^{b}	0.40^{c}	0.41^{b}	0.39^{c}	0.55^{a}	0.43^{b}
(±SE)	*±0.02*	*±0.02*	*±0.01*	*±0.04*	*±0.02*	*±0.01*	*±0.01*	*±0.02*	*±0.07*	*±0.02*	*±0.04*	*±0.03*	*±0.01*	*±0.01*	*±0.02*	*±0.03*	*±0.02*	±0.01
GLY	0.24^{de}	0.55^{a}	0.26^{de}	0.56^{a}	0.29^{d}	0.50^{a}	0.29^{d}	0.49^{a}	0.48^{a}	0.35^{c}	0.51^{a}	0.43^{b}	0.50^{a}	0.53^{a}	0.19^{e}	0.51^{a}	0.40^{b}	0.35^{c}
(±SE)	*±0.01*	*±0.02*	*±0.01*	*±0.01*	*±0.02*	*±0.03*	*±0.03*	*±0.03*	*±0.05*	*±0.02*	*±0.01*	*±0.03*	*±0.02*	*±0.04*	*±0.01*	*±0.04*	*±0.08*	*±0.01*
THR	0.87^{e}	1.42^{bc}	0.61^{f}	1.36^{c}	0.93^{e}	1.38^{c}	0.94^{e}	1.35^{c}	1.62^{a}	0.72^{f}	1.52^{b}	0.91^{e}	1.36^{c}	1.34^{c}	0.68^{f}	1.18^{d}	1.63^{a}	1.41^{bc}
(±SE)	*±0.03*	*±0.08*	*±0.09*	*±0.04*	*±0.01*	*±0.03*	*±0.08*	*±0.06*	*±0.17*	*±0.07*	*±0.02*	*±0.13*	*±0.09*	*±0.02*	*±0.03*	*±0.02*	*±0.12*	*±0.08*
ALA	0.08^{c}	0.28^{a}	0.08^{c}	0.24^{a}	0.11^{c}	0.24a	0.13^{bc}	0.24^{a}	0.23^{a}	0.11^{c}	0.27^{a}	0.14^{bc}	0.25^{a}	0.24^{a}	0.08^{c}	0.18^{b}	0.18^{b}	0.20^{ab}
(±SE)	*±0.01*	*±0.01*	*±0.01*	*±0.01*	*±0.01*	*±0.02*	*±0.01*	*±0.01*	*±0.03*	*±0.01*	*±0.01*	*±0.03*	*±0.03*	*±0.01*	*±0.01*	*±0.01*	*±0.02*	*±0.02*
TYR	0.17^{de}	0.17^{de}	0.41^{c}	0.39^{c}	0.27^{cd}	0.24^{d}	0.23^{d}	0.34^{c}	0.28^{cd}	0.56^{ab}	0.23^{d}	0.59^{a}	0.34^{c}	0.37^{c}	0.14^{e}	0.65^{a}	0.62^{a}	0.53^{b}
(±SE)	*±0.00*	*±0.02*	*±0.05*	*±0.02*	*±0.02*	*±0.01*	*±0.02*	*±0.03*	*±0.04*	*±0.06*	*±0.01*	*±0.04*	*±0.04*	*±0.03*	*±0.01*	*±0.07*	±0.03	*±0.03*

CYS	0.08^{a}	0.03^{c}	0.04^{c}	0.04^{c}	0.07^{ab}	0.04^{c}	0.04^{c}	0.09^{a}	0.07^{ab}	0.04^{c}	0.05^{bc}	0.06^{abc}	0.09^{a}	0.08^{a}	0.07^{ab}	0.07^{ab}	0.05^{bc}	0.06^{abc}
(±SE)	*±0.01*	*±0.01*	*±0.01*	*±0.01*	*±0.01*	*±0.01*	*±0.01*	*±0.01*	*±0.01*	*±0.01*	*±0.01*	*±0.01*	*±0.01*	*±0.01*	*±0.01*	*±0.01*	*±0.01*	*±0.01*
VAL	0.19^{f}	0.71^{a}	0.17^{fg}	0.66^{a}	0.23^{e}	0.59^{b}	0.21^{e}	0.67^{a}	0.46^{c}	0.29^{d}	0.63^{ab}	0.43^{c}	0.61^{ab}	0.59^{b}	0.15^{g}	0.57^{b}	0.68^{a}	0.65^{a}
(±SE)	*±0.01*	*±0.02*	*±0.02*	*±0.02*	*±0.01*	*±0.02*	*±0.02*	*±0.02*	*±0.04*	*±0.02*	*±0.02*	*±0.07*	*±0.06*	*±0.01*	*±0.01*	*±0.02*	±0.02	*±0.02*
MET	0.19^{g}	0.61^{bc}	0.17^{g}	0.55^{c}	0.24^{f}	0.52^{c}	0.26^{f}	0.54^{c}	0.52^{c}	0.29^{f}	0.57^{c}	0.36^{e}	0.52^{c}	0.52^{c}	0.14^{h}	0.46^{d}	0.67^{b}	0.76^{a}
(±SE)	*±0.01*	*±0.02*	*±0.02*	*±0.02*	*±0.01*	*±0.02*	*±0.03*	*±0.02*	*±0.05*	*±0.03*	*±0.03*	*±0.05*	*±0.04*	*±0.01*	*±0.01*	*±0.02*	*±0.02*	*±0.06*
TRP	0.26^{e}	0.28^{e}	0.35^{d}	0.23^{f}	0.61^{b}	0.17^{f}	0.06^{h}	0.15^{fg}	0.64^{b}	0.09^{h}	0.15^{fg}	0.27^{e}	0.32^{de}	0.43^{c}	0.17^{f}	0.12^{g}	0.10^{h}	0.89^{a}
(±SE)	*±0.01*	*±0.01*	*±0.01*	*±0.01*	*±0.05*	*±0.01*	*±0.01*	*±0.01*	*±0.02*	*±0.01*	*±0.01*	*±0.01*	*±0.02*	*±0.04*	*±0.01*	*±0.01*	*±0.01*	*±0.04*
PHE	0.23^{e}	0.76^{a}	0.22^{e}	0.76^{a}	0.28^{d}	0.65^{b}	0.31^{d}	0.63^{b}	0.61^{b}	0.29^{d}	0.68^{ab}	0.45^{c}	0.64^{b}	0.63^{b}	0.16^{f}	0.59^{b}	0.74^{a}	0.49^{c}
(±SE)	*±0.01*	*±0.02*	*±0.04*	*±0.03*	*±0.01*	*±0.01*	*±0.03*	*±0.05*	*±0.06*	*±0.04*	*±0.03*	*±0.07*	*±0.08*	*±0.01*	*±0.02*	*±0.03*	*±0.02*	*±0.02*
ISO	0.06^{hi}	0.57^{c}	0.08^{h}	0.48^{d}	0.08^{h}	0.52^{cd}	0.12^{g}	0.61^{c}	0.34^{e}	0.22^{f}	0.53^{cd}	0.35^{e}	0.51^{cd}	0.52^{cd}	0.04^{i}	0.49^{d}	0.96^{a}	0.81^{b}
(±SE)	*±0.01*	*±0.02*	*±0.02*	*±0.03*	*±0.01*	*±0.03*	*±0.01*	*±0.03*	*±0.04*	*±0.04*	*±0.03*	*±0.08*	*±0.08*	*±0.02*	*±0.01*	*±0.04*	*±0.05*	*±0.09*
LEU	0.63^{f}	1.32^{b}	0.52^{g}	1.31^{b}	0.63^{f}	1.22^{c}	0.58^{fg}	1.18^{cd}	1.13^{d}	0.54^{g}	1.28^{b}	0.62^{f}	1.18^{cd}	1.16^{cd}	0.29^{h}	1.04^{e}	1.40^{a}	1.28^{b}
(±SE)	*±0.07*	*±0.07*	*±0.08*	*±0.02*	*±0.02*	*±0.05*	*±0.06*	*±0.06*	*±0.10*	*±0.07*	*±0.04*	*±0.06*	*±0.10*	*±0.02*	*±0.02*	*±0.02*	*±0.07*	*±0.04*
LYS	0.48^{h}	1.27^{c}	0.38^{i}	1.06^{d}	0.49^{h}	1.21^{c}	0.53^{h}	1.14^{cd}	1.19^{c}	0.46^{h}	1.25^{c}	0.63^{g}	0.89^{e}	0.92^{e}	0.39^{i}	0.74^{f}	1.52^{a}	1.36^{b}
(±SE)	*±0.04*	*±0.04*	*±0.06*	*±0.03*	*±0.02*	*±0.10*	*±0.02*	*±0.02*	*±0.11*	*±0.01*	*±0.08*	*±0.03*	*±0.02*	*±0.04*	*±0.02*	*±0.09*	*±0.08*	*±0.07*
HYP	0.13^{d}	0.06^{e}	0.33^{c}	0.33^{c}	0.28^{c}	0.31^{c}	0.29^{c}	0.42^{ab}	0.40^{b}	0.43^{ab}	0.39^{b}	0.43^{ab}	0.47^{ab}	0.49^{ab}	0.45^{ab}	0.54^{a}	0.46^{ab}	0.39^{b}
(±SE)	*±0.01*	*±0.01*	*±0.02*	*±0.02*	*±0.01*	*±0.02*	*±0.01*	*±0.03*	*±0.03*	*±0.02*	*±0.02*	*±0.04*	*±0.04*	*±0.03*	*±0.02*	*±0.05*	*±0.04*	*±0.02*
TAA	7.52^{i}	14.45^{c}	7.66^{i}	13.9^{d}	7.52^{i}	13.61^{de}	7.70^{i}	15.76^{b}	16.57^{a}	10.03^{g}	16.40^{a}	9.38^{h}	13.48^{e}	13.53^{de}	6.13^{j}	12.15^{f}	16.45^{a}	13.40^{e}
(±SE)	*±0.33*	*±0.04*	*±0.27*	*±0.17*	*±0.09*	*±0.37*	*±0.49*	*±0.32*	*±0.48*	*±0.22*	*±0.12*	*±0.84*	*±0.78*	*±0.45*	*±0.12*	*±0.51*	*±0.24*	*±0.23*

*Superscripts with different letters in a row are significantly different 0.05 (DO:Design order, TAA:Total Amino acids),

** Optimization procedures suggested by the RSM were performed and obtained data given in O_1 and O_2

CONCLUSION

In the view of the results, two different hydrolysing procedures were suggested for amino acid analysis in fish meat. Our results were also corrected by the RSM models. According to models, the first procedure was suggested as hydrolysing in 3.79 N at 110°C in 24 hours to get maximum total and essential amino acid contents. The second one was suggested as 3.42 N at 106.8°C in 12.02 hours for maximizing Trp-Asn-Gln which are degraded conventional digestion procedures, often. Besides, despite the acidic hydrolyzation procedure with HCl is cheap, it can be uneconomic in long-standing processes due to an increasing amount of HCl and operational costs such as electricity by virtue of constant heating. Leastwise, aforementioned costs were lessened with optimization in the study In conclusion, the mentioned hydrolyzation procedures are reliable as well as accurate and they can be used in amino acid analysis by HPLC-DAD as pre-treatment in fish meat.

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