Calpain activity and texture in Atlantic salmon muscle

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Abstract

Calpain system consists of L- calpain , m- calpain, the calcium- dependent neutral proteases, and their endogenous inhibitor , calpastatin. Calpain system is probably the major proteolytic enzyme in protein degradation, which plays an important role in myofibrillar protein degradation, so the activity of calpain system is expected to have an effect on muscle texture(Jin Haili et al., 2012). This paper reviews the structure, function and regulation of calpain system, and presents the significant differences mean value of calpain activity between families, so does the texture break force. And then tells about the correlation between calpain activity and muscle texture .

Key words

Calpain activity, Texture, Break force, Atlantic salmon

Contents

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Ås, July, 2013

Abbreviations

ADP **Adenosine diphosphate**

ATP **Adenosine triphosphate**

DEAE **Diethylaminoethyl**

EDTA **Ethylenediaminetetraacetic acid**

EGTA **Ethylene glycol tetraacetic acid**

F_{break} **Breaking force**

F_{max} **Force at maximum compression**

N **Newton**

SDS **Sodium Dodecyl Sulfate**

TCA **Trichloroacetic acid**

1. Introduction

1.1 Atlantic salmon industry in Norway

According to the fisheries statistics report released by the UN Food and Agriculture (FAO), the global commercial valued salmon are more than 30 varieties, but the most common breeding species are four kinds of salmon (Pacific salmon, Coho salmon, Atlantic salmon, Arctic white spots salmon) and trout (rainbow trout, three text trout). The global aquaculture industry is continuously growing, and today it is one of the leading industries in many countries, especially in Norway. In 2009, Norway produced over 860 000 tons of Atlantic salmon and 76 000 tons of rainbow trout(Ministry of fisheries and costal affairs, 2010).

1.2 Living environment of Atlantic salmon

Under natural conditions, salmon like habitat in clear and pollution-free waters, its life limited to temperatures between 0 ℃ and 30 ℃, the optimum temperature for growth is 16 to 18 °C(Luo Gang, 2009). Best appetite and rapid growth within a temperature range. When the water temperature drops below 8 °C or higher than 20 ℃, salmon loose appetite, which also lead to growth reduction. Salmon likes the countercurrent and aerobic water environment, where they can keep good metabolism. Therefore, the salmon farming always use flow water, appropriate water flow rate is from 2 to 30 cm / s, dissolved oxygen demanding the suitability of dissolved oxygen in the amount of 6 mg / L or more. The optimum pH range is 6.5 to 7.5. Salmon is a carnivorous fish, small trash fish and aquatic insects can be the normal food in natural waters(Huang Zhiqiu, 2006).

With the considerable increase of aquaculture industry, farmed fish, especially salmon, has raised several challenges known as salmon lice, escaping, disease control, and product quality. To meet these challenges The Norwegian Research Council is

financing research projects to procure basic knowledge about which factors in farming, slaughter, handling and processing that affects the nutritional and sensory quality of farmed seafood(Mari Gaarder et al., 2011).

1.3 Muscle structure

In vertebrates, three muscle types are present: smooth muscle, cardiac muscle and skeletal muscle. It is the skeletal muscle which is considered as the muscle that is referred to as meat post mortem. The skeletal muscle constitute 50% of the body weight in pelagic fish species(Lynum, 1996). This muscle is mainly composed of myofibrillar protein, and the myofibrillar content of the total protein content is higher in fish(60-80%) than in mammals(40%)(Delbarre-Ladrat et al., 2006)

1.4 Texture analyses

Texture was analysed by a texture analyser, model TA-XT2 (SMS, Stable Micro Systems; Surrey, England), equipped with a Warner–Bratzler blade. A computer using the Texture Expert Ver.1.0 software from SMS was used to operate the instrument. In the front and the tail of the fillet, cylindrical longitudinal muscle samples from the epaxial part of the fillet were cut out with a borer of 11 mm in diameter (Fig. 1). The muscle samples were kept wrapped in plastic in a cold store, 6±1 ℃, until one measurement per muscle sample was made. In case of an atypical power curve, the measurement was repeated.

The Warner–Bratzler blade was pressed down at a constant speed of 2 mm/s through the sample, cutting the muscle fibre transversely. Maximum shear force (N) and total force (N) were measured. They are the maximum resisting (toughness or break point) and total forces needed to cut the sample, respectively. These two measurements represent the texture of the fillet and were run in parallel in the statistical evaluation.

Because both gave the same overall results and maximum shear force gave the most consistent data, it is used in the present paper as the primary measurement of the fillet softness.

1.5 Calpain system

The calpain system is widely found in livestock and human tissue. Normally, it plays a very important role in integrity of the structure and function of the maintenance of various types of cells. With the development of molecular biology techniques and related technologies, such as auxiliary markers selective breeding (MAS) technology, the calpain, and calpain inhibitor protein genes can be used as molecular markers for meat tenderness. This can again, produce more livestock and poultry meat products. Therefore recombinant DNA technology can also be used, over Calpastatin expression, to achieve controlling of the degradation of myofibrillar accelerate protein deposition objectives. On the other hand, according to the study of physiological characteristics and their pathological role of calpain, produce similar as calpain inhibition of calpain inhibitor protein, can be a solution for a variety of diseases, such as cataracts, cancer.

1.6 Calpain activity in muscle

It has been found that changes in muscle tissue of the biological characteristics (such as muscle fibers, collagen, fat, and enzymes) can affect meat quality, especially muscle fiber properties. Muscle growth rate depends on three factors: the number of muscle cells, muscle protein synthesis and degradation speed. The number of muscle cells mainly degradation by the speed of the genetic traits that embryonic muscle protein. Muscle protein degradation rate Relatively lower growth rate would result in an increase of muscle will provide high intake of nutrients to the muscles of conversion efficiency(Cui Yan et al,. 2010).

Myofibrillar protein is the main protein component of skeletal muscle, accounting for 50% to 60% of the total amount of mature skeletal muscle protein. Muscle protein

degradation pathways are mainly the three: lysosomal cathepsin pathway, pathways and ATP calpain protein metabolic pathways.

The first step in the degradation of myofibrillar degradation assembled into filaments, which may be myofibrillar protein degradation rate-limiting process steps. Numerous studies have shown that the calpain system is involved in this too adjustment process, degradation of the desired filaments only by a special cut of calcium proteasome system. Skeletal troponin enzyme present in the interior of cells in the Z-line The highest concentration. In vitro tests showed myofibrillar pure μ-calpain Or m-calpain in the presence of Ca2 + and cultured under conditions, Z Line completely disappeared, myofibrils released filaments in calpain (Bice T et al,. 2003).

The presence of inhibitors, the release of muscle fiber ratio. Calcium Protein Enzyme myofibrillar protein degradation triggered the possible mechanism is as follows: calcium Protease Z line (the thin filaments anchor in the myofibrils) and muscle Associated protein, with actin (the thick filaments and thin filaments fixed in Myofibrils) degradation of myofibrils released filaments. Thin filaments of Myofibril protein and tropomyosin and thick filaments of C-protein drop Solution. Thick filaments and thin filaments were dissociated myosin and actin protein, releasing thick filaments and thin filaments with the parent or other Myofibril reassembly also be cytoplasmic or lysosomal proteases Cathepsin degraded into amino acids; myofibrils stub fully functional, contraction strength weakened. By calpain system Regulating muscle development, thereby enhancing lean and improve meat quality. Kristensen and other research indicates, m-calpain involving pork Growth(Yang Xiao-jing, et al,. 2009). It was found that injection of recombinant growth hormone for pigs Su, the longissimus dorsi muscle calpain 3 mRNA expression of a rising trend Potential, semitendinosus muscle calpain 3 mRNA relative abundance significantly on L, in the process of muscle growth may also be involved in calpain 3 eggs Degradation of the white matter.

2. Materials and methods

This experiment used several different kind of methods for purification and activity measurement of the calpains (Camou et al,. 2007; Geesink and Koohmaraie 1999c; Geesink et al,. 2005b). It is quite common to use column chromatography to purify the calpains and then quantify the activity with assays. These methods, however, requires several steps and is time consuming. In addition to column chromatography, casein zymography is widely used to detect calpain activity (Raser et al,. 1995; Veiseth et al,. 2001). The above mentioned methods are time consuming, and kits for calpain activity measurement have been developed.

2.1 Sampling of Atlantic salmon

The salmon samples are taken from Averøy Norway, October 2012. We slaughtered around 100 salmon to get their fillet. Then the fillets were pre-rigor and stored on ice.

2.1.1 Texture Analysis-Force test

Fillet texture was measured instrumentally at 5 days post-mortem. Significant differences in fillet texture were found between families, and the loss of fillet firmness also varied significantly between families.

We measured weight and length for whole fish, and weight, PH, temperature, Fbreak, Max Force for the fillets.

2.2 Extraction

The m-calpain enzyme was purified for the first time from pork muscle several years ago(Dayton et al. 1976), however, it is still a hard job to purify calpain and separate it from its inhibitor, calpastain. The most important calcium chelator in the buffer is

EDTA or EGTA.

2.2.1 Homogenization of muscle samples

A relatively small number were taken from the freezer each time(12), and kept on ice. Small pieces of each muscle sample were cut out, and weighted very carefully in 300 mg(297-303) into the special homogenization tube. The weight for each sample was registered. 900 micro-liter of cold extraction buffer (containing DTT) added.

The samples were then homogenized at 6500 for 20 seconds (two times) and then centrifuged using the table centrifuge at 13000 rpm for 30 minutes at 4℃.

The supernatant was then transferred (by pipetting) to new tubes, and the volume measured. If the samples had to be frozen, they were divided into three tubes and frozen on liquid nitrogen before storing them at -80℃ (If the total volume is less than 500 micro-liter the whole process had to be repeated).

2.2.2 Buffer

Weigh in 6.1 gram of Trizma base, 1.86 gram of EDTA, then adjust pH to 8.3, fill up with water to 500 ml. This buffer is stable and can be stored in the fridge. When using the buffer, 1 micro-liter of 1M DTT has to be added per milliliter buffer.

2.3 Gel

Set up the gel flat with shelf, layer gels about 5.5 cm high. In cassettes 6 ml solution added, also a layer of water-saturated butanol (a mixture of 50% dest. water and 50% butanol) added. Let the gels polymerize in about 1 hour then use dest. water to remove butanol residues. Then use the filter to draw water residue.

Making 4 gels solution:

1.5 M Tris-HCL, ph 8.8	6.25 ml
dd H2O	625 µl
Casein solutions	7.5 ml

30% acrylamide(37.5:1)	10.4 ml
10% APS	125 µl
TEMED	12.5 µl

Layer stacking gels with the desired number of wells. Allow stacking gels polymerize for about 1 hour or put straight into the refrigerator if not used the same day. Use water to remove any remaining in the wells after well combs is removed. Gels are best for using when they are new, but can be stored 2 days in a refrigerator.

Making 4 gels solution:

0.5 M Tris-HCL, ph 6.8	1.88ml
dd H2O	4.63ml
30% acrylamide(37.5:1)	1.0ml
10% APS	50µl
TEMED	7.5µl

①casein-solutions: 7 mg casein/ml in 100mM Tris, adjust pH to 8.8, Sprinkle very gently under weak agitation, this takes a long time. Keep refrigerated.

②100mM Tris-HCL pH 8.8: 6.1 g Trisma Base add water until 500ml, adjust pH with HCL until 8.8, keep in fridge.

③1.5M Tris-HCL pH 8.8: 181.7 g add water to 1000ml, adjust pH with HCL until 8.8, keep in fridge.

④10%APS: 100 mg Ammonium Persulfat add 1 ml water, can be maximum kept 1 week in fridge.

⑤5M Tris-HCL ph 6.8: 30.3 g Trisma Base to 500ml water, adjust pH to 6.8, keep in fridge.

⑥1M DTT: 15.4 g DTT put in 100ml pure water, keep in the freezer with -20℃.

2.4 Electrophoresis and solutions

2.4.1 Sample Preparation

Sample preparation must be done the same day as you drive electrophoresis. mixed sample and sample buffer gently by pipetting up and down a few times.

Calculate the dilution of the sample so that 1 ul = 0.1 mg muscle and total volume of 500 ul and dilute the sample in sample buffer.

A. concentration in the supernatant(C)

C=mg muscl/ul supernatant

B. volume of supernatant(V) V=100 ul*0.1mg/ul/(C)

C. volume of sample buffer(X) X=100 ul - V

Sample buffer(20 ml):

0.5 M Tris, ph 6.8	6ml
87%Glycerol	4.6ml
8 mg/ml bromophenol blue	0.2 ml
dd H2O	9.2 ml

2.4.2 Electrophoresis

①Assemble equipment.

②Remove the cams in gels, rinse with dd H_2O and remove white tape on the bottom of the gel.

③Fill the inner chamber with electrophoresis buffer and fill the outer chamber with the rest of the buffer and the chamber is full. Full outer chamber will probably help keep the gels cool while driving.

④Let gels run at 100 volts for 15 min before the samples added to the wells. Everything happens on cold room 4℃.

⑤Apply 10 ul sample in each well. Standard in the first and last well, each sample in 2 wells.

⑥Electrical Forer: 100 volts for 4 hours at refrigerated.

⑦After elekroforesen gels taken out of the plates (remember to take off the corner of the homepage) and incubate incubation buffer:

3 * 20 min in 50 ml of incubation buffer at 4℃.

17 hours in 100 ml buffer at 4℃.

⑧The gels stained with Coomassie blue (R-250) for 1 hour at room temperature, 50 ml per gel.

⑨The gels decolorized for 2 hours at room temperature, 100 ml avfargingsløsning per gel.

⑩The gels is then in dd H2O until scanning.

2.4.3 Solutions

10 * electrophoresis buffer (2L):

Tris(Trizma base, 12114g/mol)	60.57g
Glycine(75.1g/mol)	288.38g
EDTA(Titriplex, 372.24 g/mol)	7.44g

This is dissolved in 1.5L dd H2O and the pH adjusted to 8.3 with 6 M HCl. Water added up to 2000ml and cooling down to 4℃ before using.

Using: dilute electrophoresis buffer 1:10 and add DTT

150 ml 10 * buffer

1.5 ml 1M DTT

Adjust the volume up to 1500 ml with H_2O

2.5 Scanning

We use Epson scan machine, set professional mode: Film w / film area guide; positive film;16 bit greyscale;300 dpi.

Place the gel on the surface, do preview, and select san area to storage. At last, type TIFF， get images of the gels.

2.6 Analysis of calpain

Using software called ImageQuant to calculate the total amount of calpain contains in

salmon muscle samples.

Found the ratio between each sample and the standard to express the activity of calpain activities in each sample.

Grand up all the results according to their families. While there are 10 families of salmon samples in total and 10 different individual(roughly) in each family.

Calpain activity was then expressed as done by Veiseth et al.(2001), with the density from each band relative to the density of the standard within each gel.

2.7 Data analysis

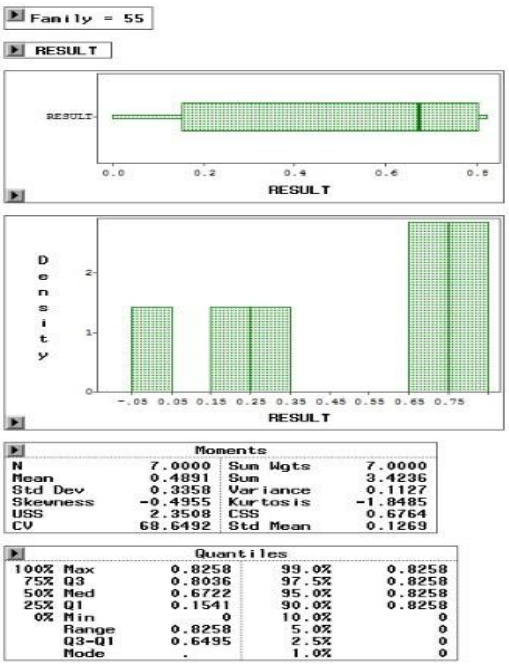
Use SAS9.2 software.

Attachment 1 presented all the data from this experiment.

3. Results

3.1 Mean of calpain activity for each family

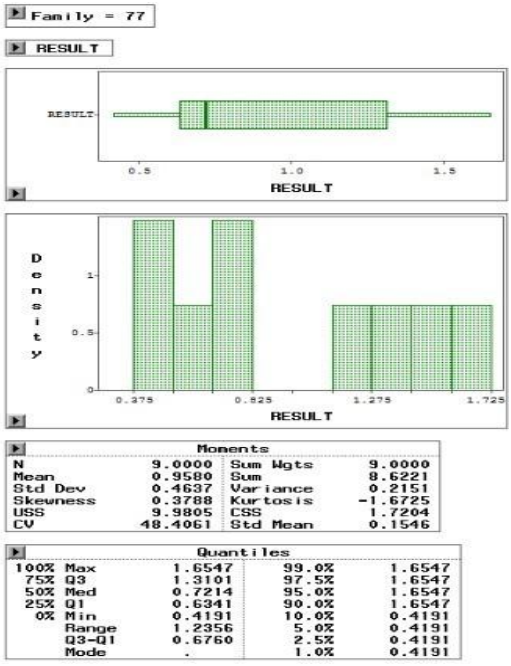
The mean value of calpain activity from each family and the comparison is presented below.



Moments			
N	7.0000	Sum Wgts	7.0000
Mean	0.4891	Sum	3.4236
Std Dev	0.3358	Variance	0.1127
Skewness	-0.4955	Kurtosis	-1.8485
USS	2.3508	CSS	0.6764
CV	68.6492	Std Mean	0.1269

Quantiles			
100%	Max	0.8258	99.0%	0.8258
75%	Q3	0.8036	97.5%	0.8258
50%	Med	0.6722	95.0%	0.8258
25%	Q1	0.1541	90.0%	0.8258
0%	Min	0	10.0%	0
	Range	0.8258	5.0%	0
	Q3-Q1	0.6495	2.5%	0
	Mode	.	1.0%	0

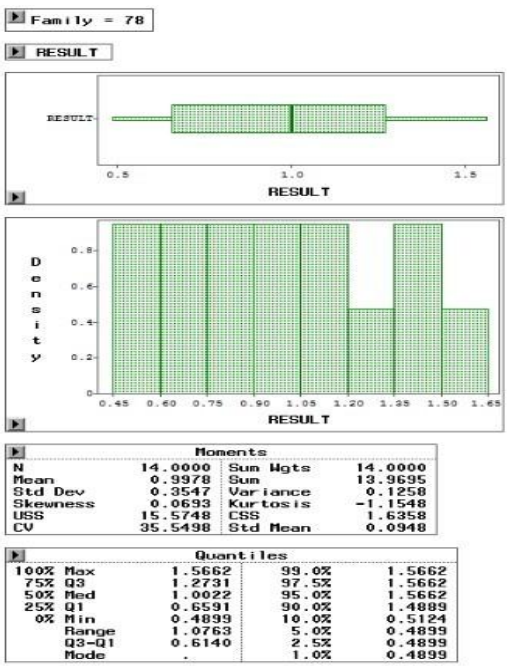
Graphic 1



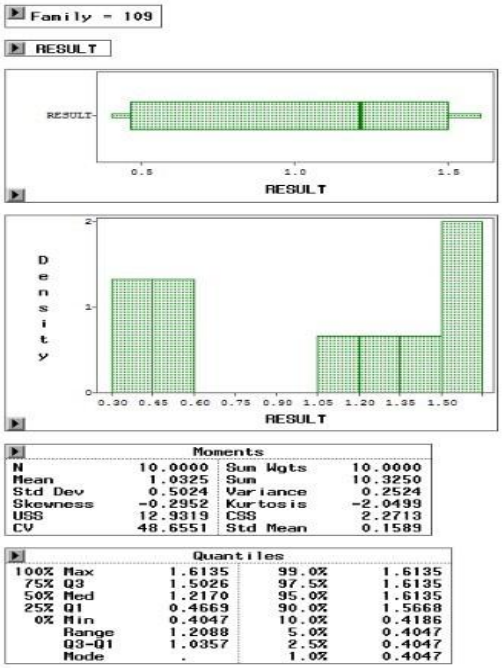
Moments			
N	9.0000	Sum Wgts	9.0000
Mean	0.9580	Sum	8.6221
Std Dev	0.4637	Variance	0.2151
Skewness	0.3788	Kurtosis	-1.6725
USS	9.9805	CSS	1.7204
CV	48.4061	Std Mean	0.1546

Quantiles			
100%	Max	1.6547	99.0%	1.6547
75%	Q3	1.3101	97.5%	1.6547
50%	Med	0.7214	95.0%	1.6547
25%	Q1	0.6341	90.0%	1.6547
0%	Min	0.4191	10.0%	0.4191
	Range	1.2356	5.0%	0.4191
	Q3-Q1	0.6760	2.5%	0.4191
	Mode	.	1.0%	0.4191

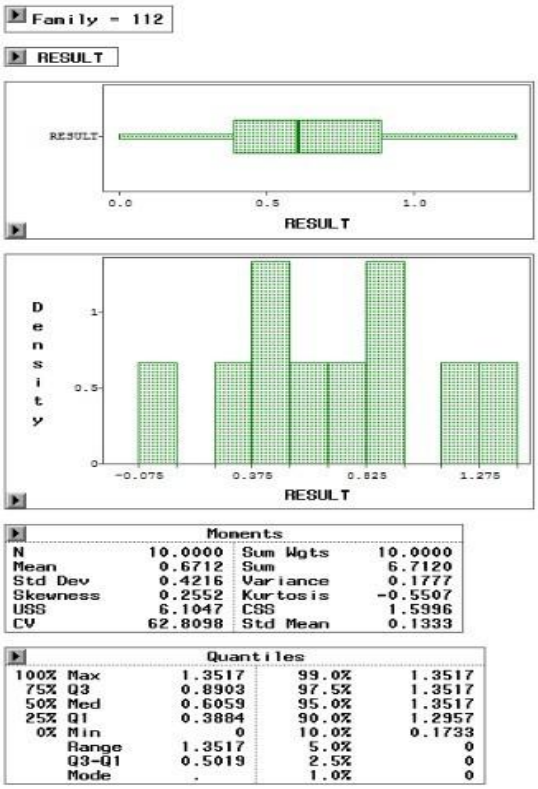
Graphic 2



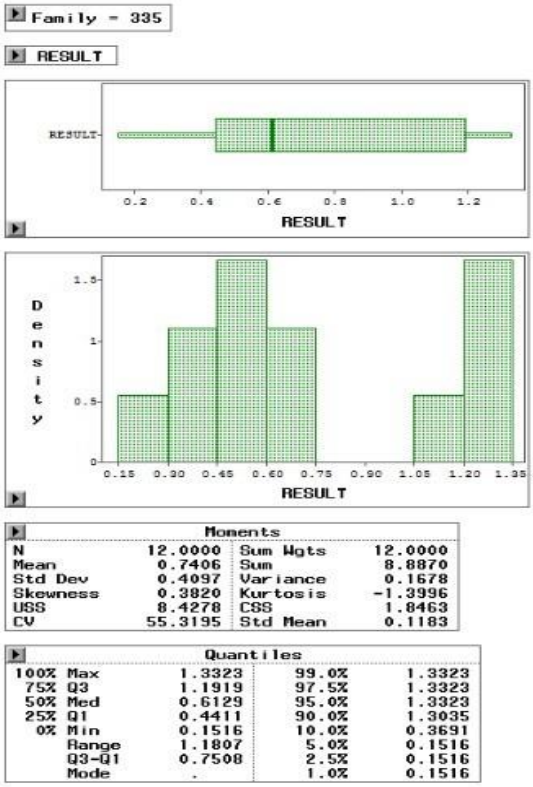
Graphic 3



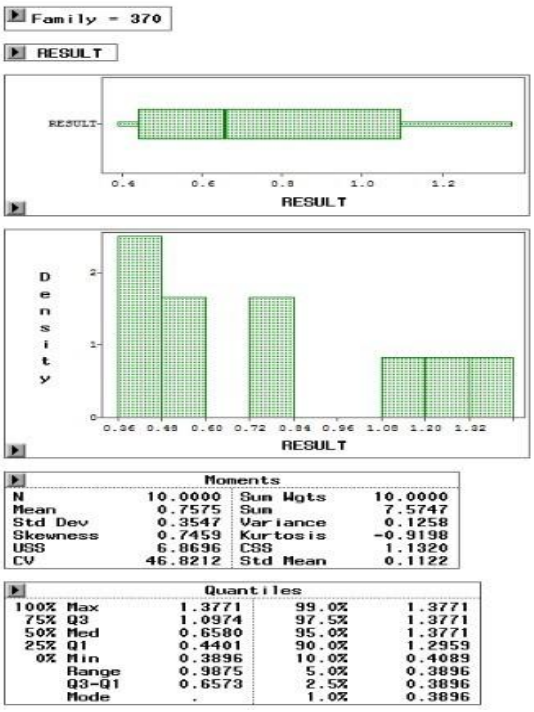
Graphic 4



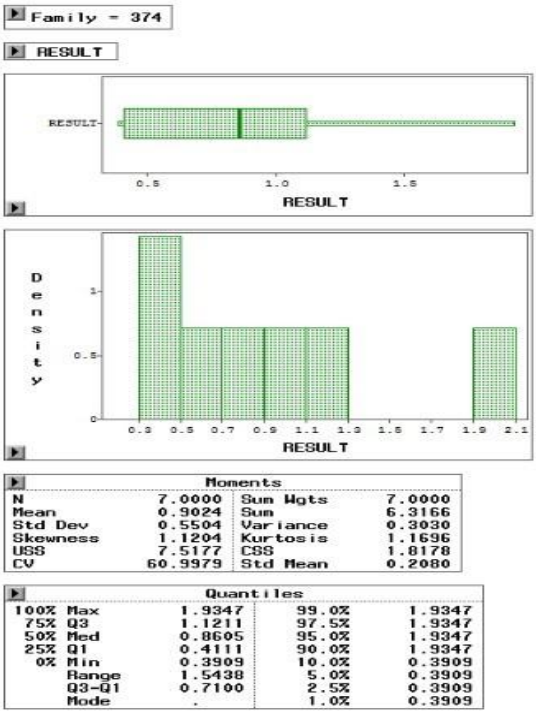
Graphic 5



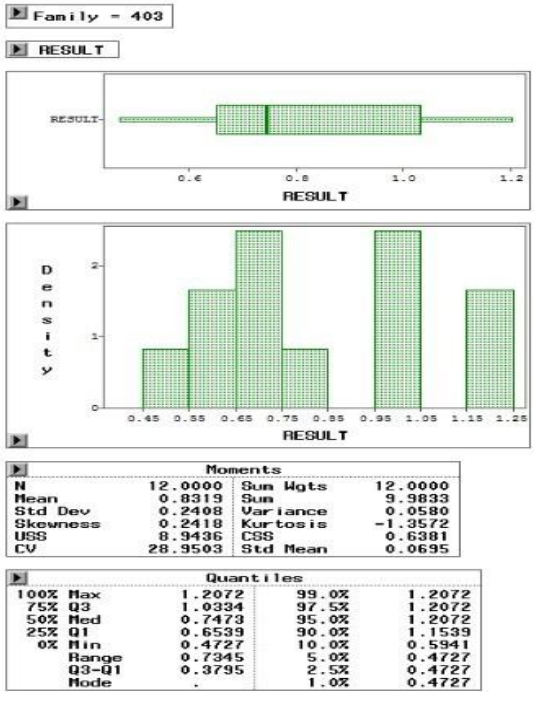
Graphic 6



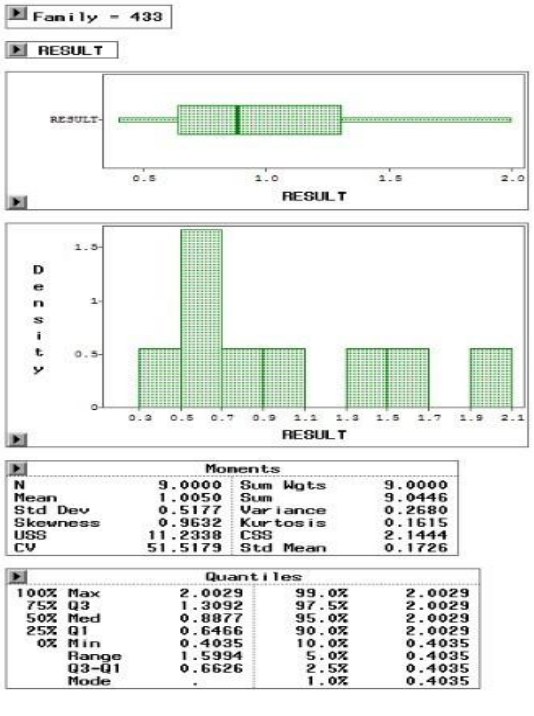
Graphic 7



Graphic 8



Graphic 9



Graphic 10

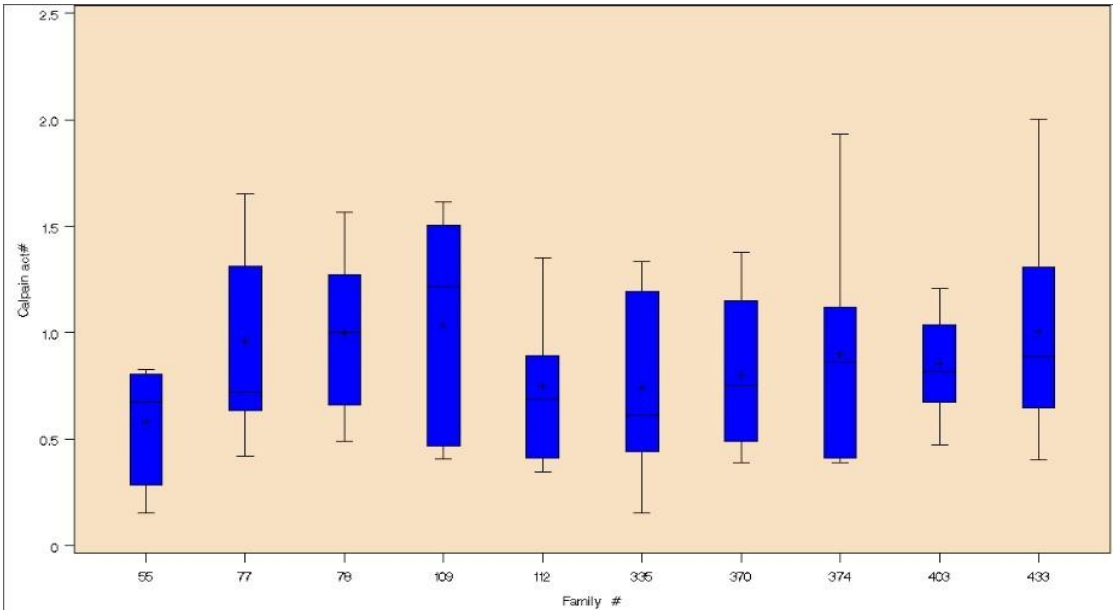
The Graphic 1 shows that the lowest mean value(0.49) of calpain activity happens in family 55, while the highest one is found in family 109(mean =1.03) as presented in graphic 4.

Mean of calpain activity from other salmon families are between 0.67 and 1.01.

3.2 **Calpain activity comparison for each family**

Here tells the differences between those 10 families for their mean values of calpain activity.

Calpain activity calculated by amount of sample divide amount of standard.

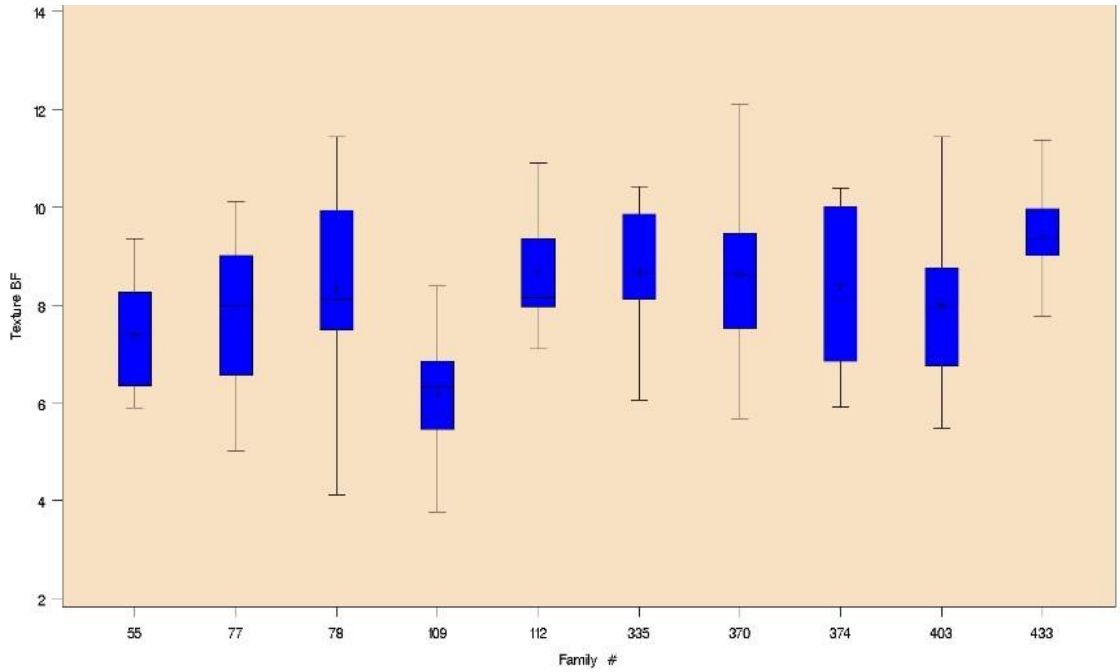


Graphic 11

Blue area from graphic 11 presents the lowest individual to the highest one.

3.3 Break Force of muscle in samples

Break force of muscle presents the tenderness of salmon muscle texture.



Graphic 12

The graphic 12 shows the differences of muscle break force between the 10 families. Lowest mean value(6.18N) of break force is found in family 109, while the highest in family 433(mean =9.41N) as illustrated in graphic 12.

Mean of break force of muscle from the other salmon families are between 7.38 and 8.69.

3.4 Correlations of mean value between Calpain activity and Texture BF

Here presents the mean value of calpain activity and texture break force in the 10 families is presented in figure 1.

Family #	Calpain(mean)	Texture BF(mean)
55	0.4891	7.3809
77	0.9580	7.9189
78	0.9978	8.3279

109	1.0325	6.1784
112	0.6712	8.6938
335	0.7406	8.6742
370	0.7575	8.6400
374	0.9024	8.3933
403	0.8319	8.0039
433	1.0050	9.4141

Table 1: Mean values of calpain activity and texture in the salmon families.

By using the SAS9.2 analyst function, the correlation between calpain and texture BF was calculated.

The CORR Procedure

2 Variables: calpain texture_bf

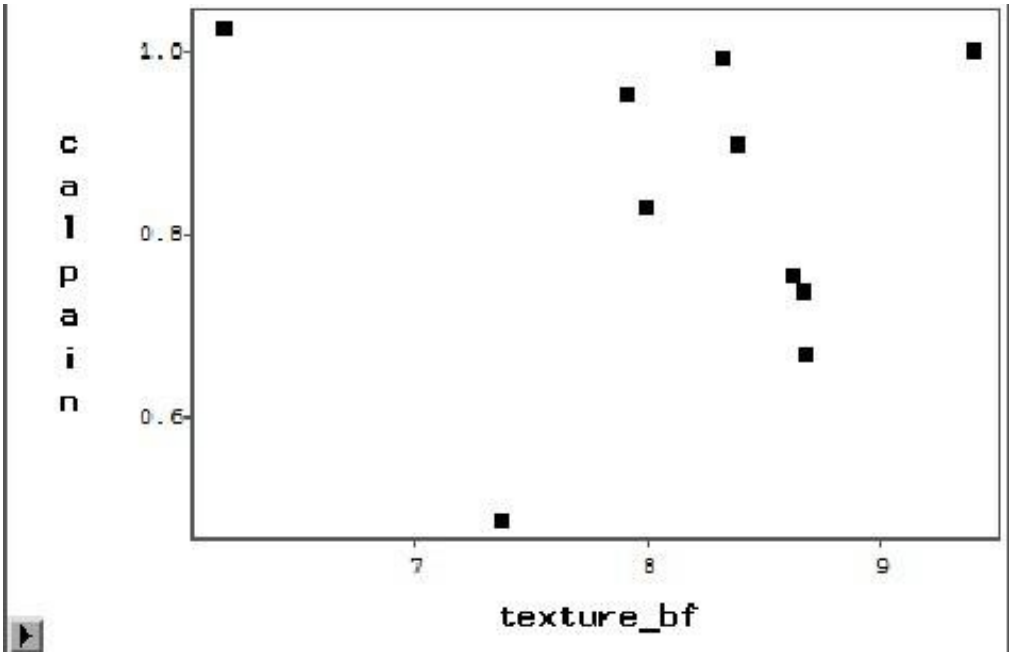
Simple Statistics

Variable	N	Mean	Std Dev	Sum	Minimum	Maximum	Label
calpain	10	0.83860	0.17491	8.38600	0.48910	1.03250	calpain
texture_bf	10	8.16254	0.88514	81.62540	6.17840	9.41410	texture bf

Pearson Correlation Coefficients, N = 10
Prob > |r| under H0: Rho=0

	calpain	texture_bf
calpain	1.00000	-0.04884
calpain		0.8934
texture_bf	-0.04884	1.00000
texture bf	0.8934	

Correlation coefficient : r = -0.04884



Graphic 13

Correlation coefficient is negative**,** indicating that mean of calpain activity and texture BF is showing inverse relationship, but the correlation coefficient is close to 0, tell in us that there is no significant correlation between the two groups of data.

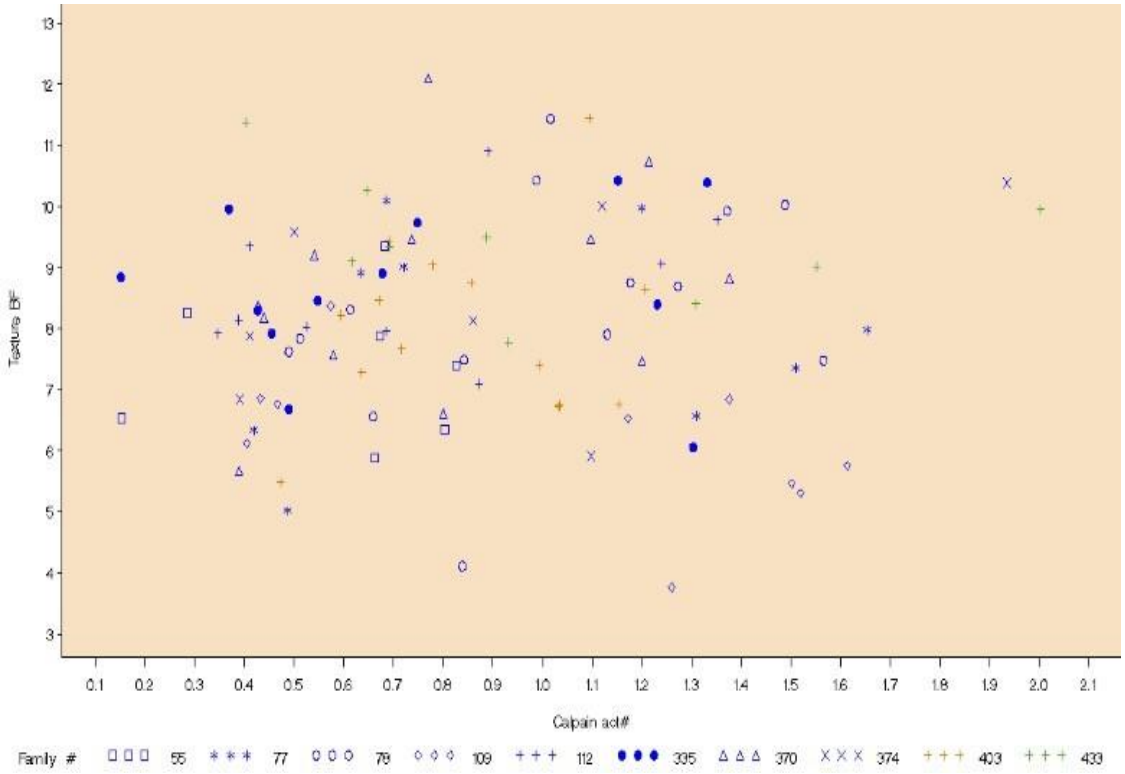
If we ignore the family 55(because it shows extremely difference to other families in the mean value of calpain activity), then we got a new correlation below:

Correlation coefficient **:** r = -0.39393

This may indicate that there exists a possible negative correlation.

3.5 Comparison of calpain activity and texture BF(All data)

When all samples' calpain activity and texture break force are compared, the following results are observed.



Graphic 14

2 Variables: Calpain_act_ Texture_BF

Simple Statistics

Variable	N	Mean	Std Dev	Sum	Minimum	Maximum	Label
Calpain_act_	103	0.86867	0.40421	89.47330	0.15160	2.00290	Calpain act#
Texture_BF	103	8.18309	1.64673	842.85800	3.76700	12.09700	Texture BF

Pearson Correlation Coefficients, N = 103
Prob > |r| under H0: Rho=0

	Calpain_ act_	Texture_ BF
Calpain_act_ Calpain act#	1.00000	0.07869 0.4294
Texture_BF Texture BF	0.07869 0.4294	1.00000

Correlation coefficient **:** r = 0.07869.

Again the correlation coefficient is close to 0, the result from SAS9.2 analyst tells that there is no significant correlation between the two groups of data.

4. Discussion

4.1 Texture instrumental measurements

In the present study, we get significant different mean value of texture break force among families, it seems to be an advantage and possible to do more breeding works at texture analysis.

Instrumental texture measurements of individual Atlantic salmon from different families showed significant differences between families in breaking forces as well. That fillet shear force in rainbow trout was significantly different between strains in arguments before.

Mean value of breaking forces for families were between 6.18 and 9.41 during this study. Generally, BF values higher than 6 is considered to mean a fairly good texture of salmon muscle This means that the texture in all families were good, but still some families seem to give an even higher and better texture.

At the slaughter of the complete family material in April 2013, half a year after the present study texture were measured again in the same families as used here. Interestingly, a high correlation of texture mean values between October and April was found (M.S. Thomassen, personal information)This means it is possible to sample fish before(even long time before) slaughter and get information about families expected to give good and less good texture at slaughter.

4.2 Calpain activity

In the present study, we got significant different mean value of calpain activity among families, it is worth mentioning that one of them is extremely lower than others, which happens in family 55(mean=0.49). This may be due to significantly lower fish weight in this family (results not shown). Several other possible reasons can be human error and samples failures, etc.

Calpain is a cytoplasm major proteolytic enzyme, plays an important role in neural development, muscle growth, the signal conduction, apoptosis and cleavage of other membrane proteins (Giiler and Spira, 1998; Molin surface and CaraJ [O 11,1997).

4.3 Correlation between calpain and texture

During this study, we found significantly different mean value of calpain activity and texture Fbreak among families. Statistics however showed that the correlation between calpain activity and texture Fbreak is not obvious. This is in accordance with results presented by Bahuaud et al (2010).

As far as we know, μ-calpain is unstable and degenerates very fast. This leads to difficulties in purifying total calpain activity. Furthermore, calpain inhibitor (calpastain) also effects the expression of calpain activity (Bahuaud et al., 2010). This means that measuring only the milli-calpain its inhibitor may not give a complete picture of the importance of this enzyme in the tenderization process of the salmon muscle.

Since removing the data for the family 55, we could see some correlation between calpain activity and texture, so perhaps we can do more reaserch on finding the importance of this enzyme for texture.

5. Conclusion

In the present study, we got significant difference in texture among the 10 families of salmon samples, and also for the calpain activity mean values. No significant correlation was, however, found between calpain activity and texture.

Based on this, we cannot conclude from our study that calpain activity affects salmon texture, however, it has been shown in other fish species before. The complexity of the calpain system in influencing Atlantic salmon muscle texture is still far from resolved. We need to invest more research, improve the current method of purification of calpain, while testing to find out more factors that affect salmon muscle structure, thereby perhaps still find that calpain do function on salmon muscle structure.

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Tube	Fish nr	Family	NO.	Amount	Volume	On gel	Sample B	Ratio
1	1	335	797	300.4	750	74.9	25.1	0.7489
8	3	335	767	299.1	690	69.2	30.8	0.3691
10	6	335	764	298.6	780	78.4	21.6	0.1516
20	2	335	765	301.9	795	79.0	21.0	0.6781
33	1	335	738	300.1	730	73.0	27.0	1.3035
43	2	335	729	300.0	795	79.5	20.5	0.4898
45	2	335	794	297.6	810	81.7	18.3	1.1523
47	6	335	762	299.5	790	79.1	20.9	0.4555
67	2	335	751	298.6	845	84.9	15.1	1.2315
68	5	335	783	299.4	800	80.2	19.8	1.3323
92	4	335	717	298.2	790	79.5	20.5	0.5477
96	2	335	718	301.9	855	85.0	15.0	0.4267
2	4	109	750	302.9	875	86.7	13.3	1.5201
6	5	109	750	297.8	840	84.6	15.4	0.4324
19	6	109	746	302.8	760	75.3	24.7	0.4047
48	3	109	736	297.4	835	84.2	15.8	0.4669
66	6	109	760	298.3	815	82.0	18.0	1.6135
57	6	109	731	300.1	775	77.5	22.5	1.3769
61	2	109	773	301.9	820	81.5	18.5	0.5739
69	2	109	733	298.4	845	85.0	15.0	1.2609
72	2	109	757	300.2	825	82.4	17.6	1.1731
100	2	109	799	297.6	810	81.7	18.3	1.5026
3	1	78	741	300.9	820	81.8	18.2	1.1774
16	3	78	772	297.4	810	81.7	18.3	0.6132
17	6	78	758	302.6	770	76.3	23.7	0.4899
28	3	78	715	302.0	710	70.5	29.5	0.5124
37	3	78	744	301.0	775	77.2	22.8	0.6591
38	5	78	703	301.9	725	72.0	28.0	0.8427
50	4	78	763	297.0	785	79.3	20.7	1.0163
60	2	78	755	302.3	805	79.9	20.1	1.1311
77	4	78	778	300.6	815	81.3	18.7	1.2731
81	3	78	771	298.8	825	82.8	17.2	1.5662
84	4	78	719	300.8	780	77.8	22.2	0.8393
90	1	78	920	300.6	800	79.8	20.2	1.4889
98	3	78	801	298.9	815	81.8	18.2	0.9881
99	5	78	805	297.8	840	84.6	15.4	1.3718
4	2	370	768	300.8	850	84.8	15.2	0.4401

27	2	370	737	301.4	795	79.1	20.9	0.3896
34	5	370	716	302.2	780	77.4	22.6	0.5793
41	6	370	743	302.1	845	83.9	16.1	1.3771
55	2	370	752	298.9	850	85.3	14.7	0.7366
59	4	370	789	301.7	810	80.5	19.5	0.5416
70	5	370	800	302.2	780	77.4	22.6	1.0974
88	6	370	807	303.0	835	82.7	17.3	1.2147
91	4	370	707	301.9	670	66.6	33.4	0.4281
94	1	370	779	300.5	810	80.9	19.1	0.7702
5	4	112	722	301.0	685	68.3	31.7	0.3466
11	1	112	788	299.6	0	0	0.0	
18	1	112	790	301.2	790	78.7	21.3	0.8723
23	1	112	721	301.5	725	72.1	27.9	1.3517
31	5	112	726	299.7	770	77.1	22.9	0.8903
35	6	112	786	299.7	705	70.6	29.4	0.6861
46	6	112	714	300.8	810	80.8	19.2	0.3884
52	4	112	788	299.8	755	75.6	24.4	0.4112
53	1	112	791	302.3	825	81.9	18.1	0.5257
56	5	112	756	300.5	730	72.9	27.1	1.2397
7	2	403	713	302.3	770	76.4	23.6	0.6716
13	6	403	720	301.9	690	68.6	31.4	0.5941
14	6	403	749	297.3	840	84.8	15.2	0.6911
24	3	403	769	301.4	750	74.7	25.3	0.7161
29	4	403	732	302.1	790	78.5	21.5	0.4727
32	2	403	702	301.1	770	76.7	23.3	1.0341
39	5	403	747	299.7	805	80.6	19.4	1.0326
40	1	403	723	300.6	800	79.8	20.2	0.7785
42	5	403	793	302.0	795	79.0	21.0	0.9953
44	1	403	709	299.5	800	80.1	19.9	0.6361
64	3	403	770	301.2	825	82.2	17.8	1.1539
76	6	403	739	299.8	820	82.1	17.9	1.2072
9	6	374	792	299.2	810	81.2	18.8	0.5006
21	6	374	740	303.0	820	81.2	18.8	0.3909
25	5	374	734	300.7	800	79.8	20.2	1.1211
79	2	374	777	300.3	805	80.4	19.6	1.9347
80	6	374	782	302.3	795	78.9	21.1	1.0977
82	4	374	730	298.4	805	80.9	19.1	0.8605
86	5	374	802	299.8	790	79.1	20.9	0.4111
12	2	55	706	298.4	840	84.5	15.5	0.6828
15	4	55	711	299.8	780	78.1	21.9	0.1541

49	5	55	727	300.2	735	73.5	26.5	0
85	4	55	804	302.4	810	80.4	19.6	0.8258
87	5	55	754	301.7	845	84.0	16.0	0.8036
93	4	55	806	299.4	735	73.6	26.4	0.2851
95	2	55	705	301.2	825	82.2	17.8	0.6722
22	1	77	759	301.2	825	82.2	17.8	1.5096
36	6	77	710	302.7	785	77.8	22.2	0.4862
58	5	77	708	299.8	690	69.0	31.0	0.4191
62	1	77	745	300.3	815	81.4	18.6	0.6341
65	1	77	735	301.3	790	78.7	21.3	1.6547
73	1	77	796	298.5	820	82.4	17.6	0.6861
75	3	77	775	298.3	795	80.0	20.0	1.2008
89	5	77	748	299.2	845	84.7	15.3	1.3101
97	3	77	795	297.0	745	75.3	24.7	0.7214
26	4	433	784	297.6	720	72.6	27.4	0.4035
30	5	433	701	299.2	770	77.2	22.8	0.9318
51	2	433	766	297.5	610	61.5	38.5	0.6935
54	2	433	785	297.4	805	81.2	18.8	0.6466
71	2	433	753	303.0	795	78.7	21.3	1.3092
74	2	433	780	300.0	820	82.0	18.0	0.6173
78	5	433	798	298.0	800	80.5	19.5	2.0029
83	4	433	761	297.9	815	82.1	17.9	0.8877
101	4	433	742	302.5	855	84.8	15.2	1.5521
63	6	NO	774	302.0	840	83.4	16.6	0.9802

Amount=mg salmon sample

Volume=µl muscle solutions

On gel=µl muscle solution used for gel

Sample B=µl Sample buffer used for gel

Result=Amount of sample divide standard

All data are grouping by families' number.

Attachment 1