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# Investing the effects of preprocessing conditions on qualitative characteristics of skin gelatin of the Iranian Beluga

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**Abstract**

The current study was done for the purpose of determining optimum conditions of gelatin extraction in different conditions, pre-processing from elephant fish (Huso huso)'s skin, by applying acids (citric acid, choleric acid) and alkalis (Sodium hydroxide, Calcium hydroxide) in different densities (0.01-0.05-0.075 Normal) on extraction range, gel strength, melting point, viscosity, molecular weight distribution and color of produced gelatin samples was conducted in three repetitions. Comparing mean values of test results of different treatments was done via Dunken method in a probability level of 95 percent. Effects of all variables on all gelatin characteristics were significant ($P<0.01$). In optimum preprocessing, conditions with the 0.01 normal choleric acid and the 0.01 normal sodium hydroxide, 21.06 % as the maximum determined value for the extraction efficiency, gel strength of 297 (gr.), melting point of 33.5 degrees Celsius, viscosity of 9.05 mPa-S, chain value of 66.9 α, chain value of 28.78β, color light of 41.14 (L*), yellowness (b*) and redness (a*) were calculated to be 11.56 and -3.08 respectively.

**Keywords**: gelatin, extraction process, beluga

## Introduction

Gelatin is a string protein extracted from collagen and it is a practical polymer with extensive uses in industries such as food, materials, pharmacy and photography. Due to its unique characteristics, collagen is very practical and useful in pharmacy and food industries (Rahman, et al, 2008). Recently its use in new programs such as operator food has been growing. About 95% of commercial gelatins are obtained from mammalian resources, mainly pig and cow's skins and the remaining 5% is produced from cow and pig's bones. However, mammalian gelatin is exposed to the danger of contamination by bovine spongiform encephalopathy. Furthermore, gelatin produced from cows and pigs are not used in some foods due to religious objections. Therefore, researches regarding fish gelatin to develop methods and tap peripheral products of fish processing is very important as an alternative to mammalian resources (Gomez- Guillen and Montero, 2001). Compared to fish gelatin, mammalian gelatin is different in many characteristics. The melting point and strength of fish gelatin's gel is lower than those of mammals (Gomez-Guillen et al, 2002). Nonetheless, gelatins of many fish species show greater viscosity than those of mammals. Recently, skin gelatins of different fish species including sharks (Cho et al, 2007), skates (Cho et al, 2006), Atlantic salmons (Arnesen and Gildberg, 2007), and sturgeons (Hao, et al, 2009) have been extracted and specified. Little is known, however, concerning characteristics of gelatins extracted from commercial species such as beluga. Beluga belongs to the Acipenser genus, the biggest fish in

the Caspian Sea. This aquatic creature's main habitats are the Caspian Sea and the Black Sea. This study was designed with purpose of optimizing the pre-treatment process used for obtaining the best physical and chemical characteristics such as efficiency, viscosity, gel strength, melting point for producing gelatin from beluga's skin.

## Materials and methods

### *Preparation of beluga's skin*

The Beluga species (Huso huso)'s skin was obtained as peripheral product in frozen form with the -30°C temperature from the Babolsar Fisheries Organization and refrigerated in -20 °C in laboratory. Meat remainders stuck to skins were removed manually, thus cleaning and washing the samples. The skins were split to segments of about 1×1cm $^2$ and refrigerated in polyethylene bags with a temperature of -20 °C for subsequent use.

### *Gelatin extraction*

Gelatin was extracted using the Zhou and Regenstein (Zhou and Regenstein, 2005) method with small changes in preprocessing parameters. To remove non-collagen proteins in the soda concentration domain (0.01 normal) of 3w/v, alkaline pre-treatment processing was performed for 1 hour with three repetitions in the temperature of 4 °C. In order to remove minerals and salts from skins after washing with water, acidic pre-treatment process was done in three different concentrations of acetic acid and choleric acid (0.01-0.05-0.075 normal) of 3w/v for 1 hour with three repetitions. Gelatin was extracted from pre-treated skin in distilled water with volume ratio of 1:2.8 in 50 °C after 7 hours in a water bath. After passing through a filtering fabric, it was compressed and concentrated under vacuum. Concentrated gelatins were kept in 4 °C for the purpose of investigating characteristics. After optimum acidity was determined, alkaline pre-treatment process (soda and calcium hydroxide) was performed in concentration domains (0.01-0.05-0.075 normality) of 3w/v for one hour. Then, after water-rinsing, acid pre-treatment process was conducted using optimum acid produced from the previous stage with 3w/v for 1 hour in 4 °C. Similar to the previous stage, gelatin was extracted from pre-treated skin and kept in 4 °C for features examination.

### *Protein measurement via the Biuret method*

Protein fraction of gelatin samples was determined using the Biuret method (Gornal et al, 1949). Samples absorption readings was done via spectrophotometer (UV/Vis, 4802 Unico) in the wavelength of 540 nm, using bovine serum albumin (Tehran biochemistry, Iran) as the standard protein.

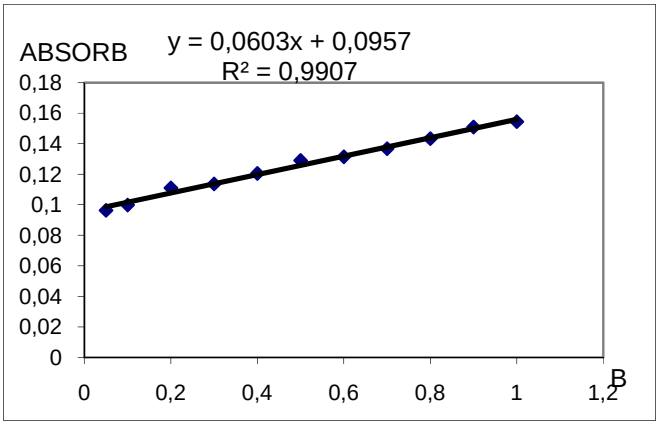


**Figure 1. Standard curve for bovine serum albumin to determine protein fraction using the Biuret method**

***Gelatin efficiency determination***

Efficiency of gelatin extraction based on filtered solution's volume of the produced gelatin was done after compression and concentration of its proteins using the following formula (Yang et al, 2007):

$$\text{Extraction efficiency percentage} = \frac{extracted\ gelatin's\ volume\ (ml) \times protein\ concentration\ \left(\frac{mg}{ml}\right)}{weight\ of\ initial\ sample\ skin\ (mg)} \times 100$$

***Gel strength determination***

Gel strength for 6.67% (W-v) gel was determined using a texture meter (CT3, LFRA Texture analyzer, Brookfield) under the following conditions: plastic cylinder piston (TA10) with 12.7 millimeters in diameter, a 1mm/s penetration speed, and a 4mm penetration depth. Maximum force in grams was recorded with a 4mm piston penetration in gel, thus reported as the gel strength (Sinthusamran et al, 2007).

***Melting point determination***

According to British standards (BS, 1974), gelatin concentration was adjusted to about 6.67% (w-v) to measure melting point. Then 4 millimeters of this solution was poured in a small test tube. The test tube was refrigerated for 12 hours (in 4-6 °C). Then, 2-3 drops of the 0.5% methyl red-chloroform were added to the samples. Next, the samples were placed in a water bath. Initial water temperature was driven to about 4 °C using ice and then the water bath was turned on and temperature rose with a rate 0.2 °C/min. As the temperature rises to gelatin's melting point, the chloroform drop starts to fall down the test tube, as will be visible due to the existence of methyl red. The temperature at which the chloroform drop fell was written on each of the samples and reported as the melting point.

***Color determination***

Color of gelatin solutions was determined by measuring values of lightness, redness, and yellowness using a HunterLab colorimetric apparatus (model Minolta Cr-300 Series, US) (Jamilah and Harvinder,2002).

***Viscosity determination***

Viscosity measurement was done using Ostwald's viscometer. The sample used for determining bloom strength of gel (6.67%) was melted in a 40 °C water tub and then poured into the viscometer. Before measuring viscosity, the viscometer was kept for 15 minutes in a 40 °C water tub.

***Electrophoresis via polyacrylamide gel***

Electrophoresis with polyacrylamide gel was done using a lower 12-percent gel and an upper 5-percent gel. Gelatin solutions (5 mg/ml) and the molecular weight standard with sample buffer was thinned with a ratio of 1:2 and then poured onto gel. After electrophoresis, the gel was soaked in Coomassie Brilliant Blue R-250 methanol, acid acetic and water with ratio of 4:1:5 (in volume terms) and then placed in methanol, acid acetic and water with ratio of 1:1:8 (in volume terms) of bleaching solution (Mohtar, et al, 2007).

***Statistical analysis***

Investigating the significance of the effect of individual treatments was done using ANOVA and means comparison was considered using Dunken's multi-domain test. SPSS software was used for data analysis and Excel was used for drawing graphs.

**Results and discussion**

Depending on pre-treatment conditions, efficiency of beluga's skin gelatin extraction in the current study ranged over 10.8-21.06 percent. Based on conducted means comparison, the best extraction efficiency was estimated to be 21.06 percent (figure 2). This number has been estimated

in skin pre-treatment conditions with 0.05-normal choleric acid and 0.01-normal sodium. Choleric acid's concentration had a significant impact on increased extraction efficiency. Choleric acid is stronger than acetic acid and in similar pH concentrations it affects the environment more, thus lowering it. Higher extraction efficiency using this acid is probably due to its effect on the hydrolysis of collagen chains and therefore better inflation and solubility of collagen compared with acid acetic. Similarly, in beluga and salmon and Alaska polloc, higher extraction range resulted in acidic pre-treatments.

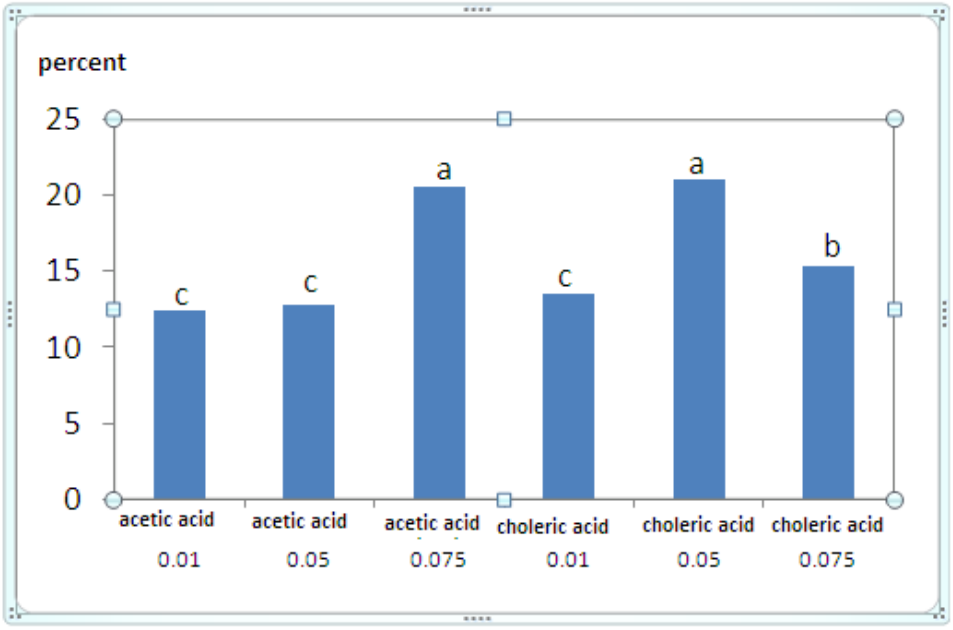


**Figure 2. Effect of acid concentration on extraction efficiency in different treatments**

Gel strength of commercial gelatins often varies between Bloom values of 100 and 300. Gelatins with higher Bloom are more desirable. In this study, Bloom strength of beluga's gelatin (6.67 % w-v) was gained over the range of 40-279 depending on pre-process conditions. According to means comparison, optimum gel strength value was estimated to be 279 (grams) (fig. 4). This value resulted in pre-process conditions of skin with 0.01 normal sodium and 0.01 normal choleric acid. Gelatin with higher alpha chain percentage shows higher gel strength (Shi, et al, 2007). Thus, achieving higher gel strength by the mentioned pre-treatment process is probably due to its role in preventing further destruction of peptide structure. Effect of 0.01-normal choleric acid on electrophoresis gel and creating protein components with higher alpha and beta chain percentages can represent an endorsement on the role of choleric acid in increasing gel strength. Gudmundsson (Gudmundsson and Hafsteinsson, 1997) showed that in pre-treatment of cod's skin with sodium and sulfuric acid solutions higher than 0.5% and citric acid higher than 1%, gel strength decreases. Beluga's gel strength in lower concentrations of sodium and choleric acid reached its maximum peak, thus showing accordance with Yang et al (2007) that had been done on catfish's gel strength. This shows that the ability to form gelatin gel is sensitive to alkaline and acidic hydrolysis because both affect collagen's lateral joints.

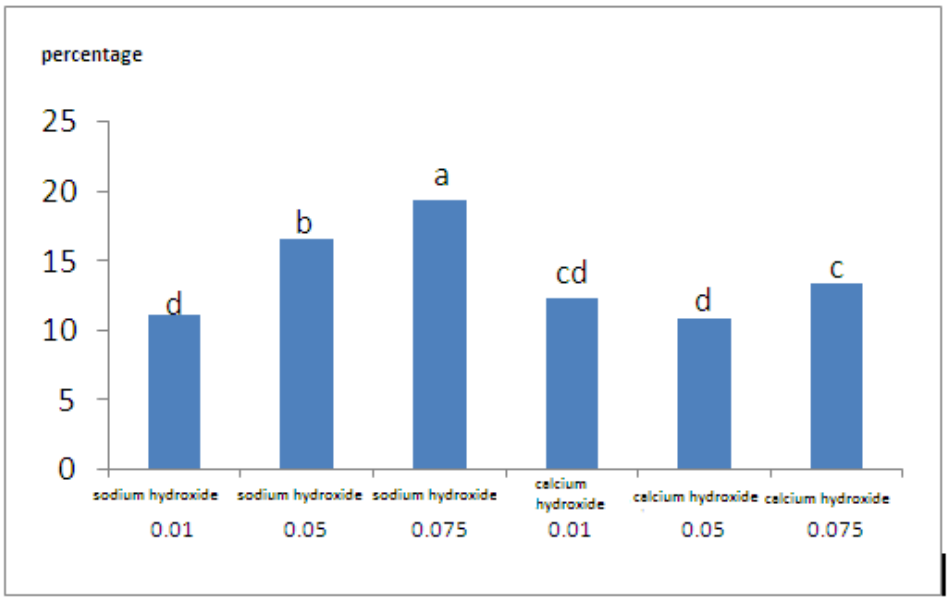


**Figure 3**. **Effect of alkaline concentration on extraction efficiency in different treatments**

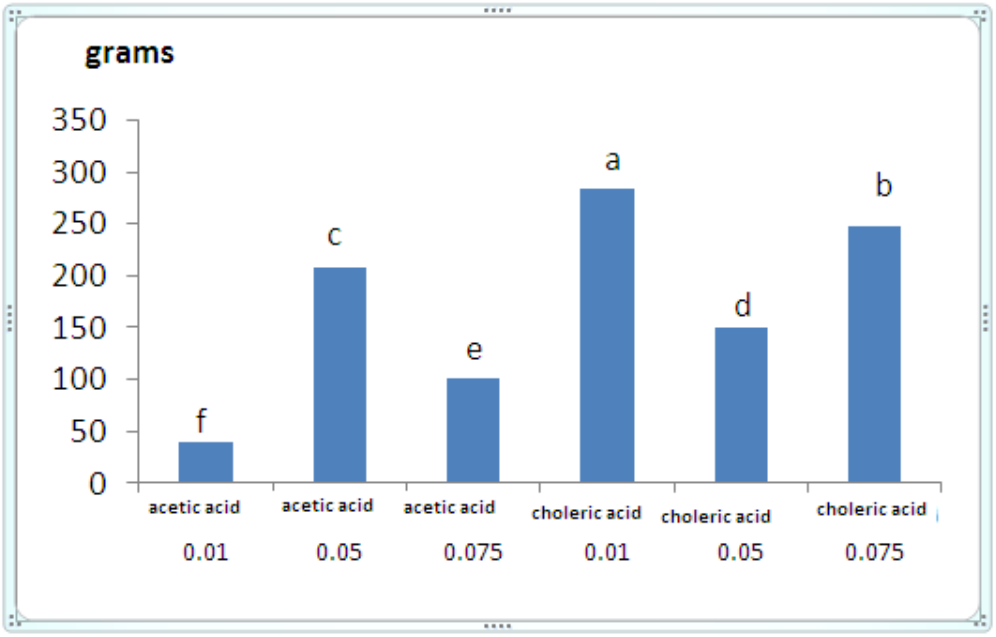


**Figure 4. Effect of acids concentration on gelatin strength in different treatments**

In various pre-process conditions, beluga's gelatin samples showed a melting point over the range of 15-33.5 °C. According to conducted means comparison, maximum melting point was estimated to be 33.5 °C (fig. 6). This value resulted in different skin pre-process conditions with 0.01-normal sodium and 0.01-normal choleric acid. Probably because it engages active factors in molecular level of chains and prevents inter-string link among them and reducing inter-chain bridges, presence of salts and impurities reduces gelatin's melting point and prevents strong gel formation. Because of being stronger and creating more hydrogen ions in its environment, 0.01-normal acid choleric has a more effective role than 0.01-normal citric acid in removing salts and

impurities, thus leading to increased melting point. Furthermore, melting point differences in different concentrations of acetic acid and choleric acid can be explained based on changes occurred in their molecular weight distribution. In concentration of 0.01-normal choleric acid, according to the pattern of beluga gelatin electrophoresis, produced gelatins have higher values of alpha and beta chains. In fact, existence of these oligomers shows gelatin's denaturation ability and reformation of ordered spiral structures and water's stabilization and orientation alongside macromolecules (Sims et al, 1997). As flexibility of gelatin molecules decreases, melting point increases. Under optimum conditions, gelatin's melting point of beluga is at most 33.5 °C higher than that of red tilapia (28.9 °C) (5), seal (28.5 °C) (16), croaker (24.57 °C) .

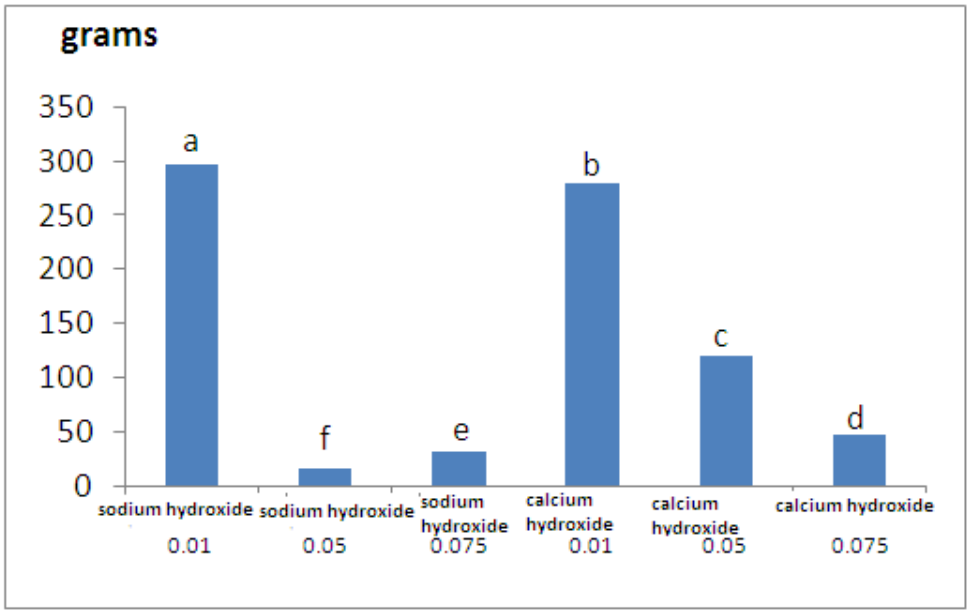


**Figure 5. Effect of alkaline concentration on gelatin strength in different treatments**

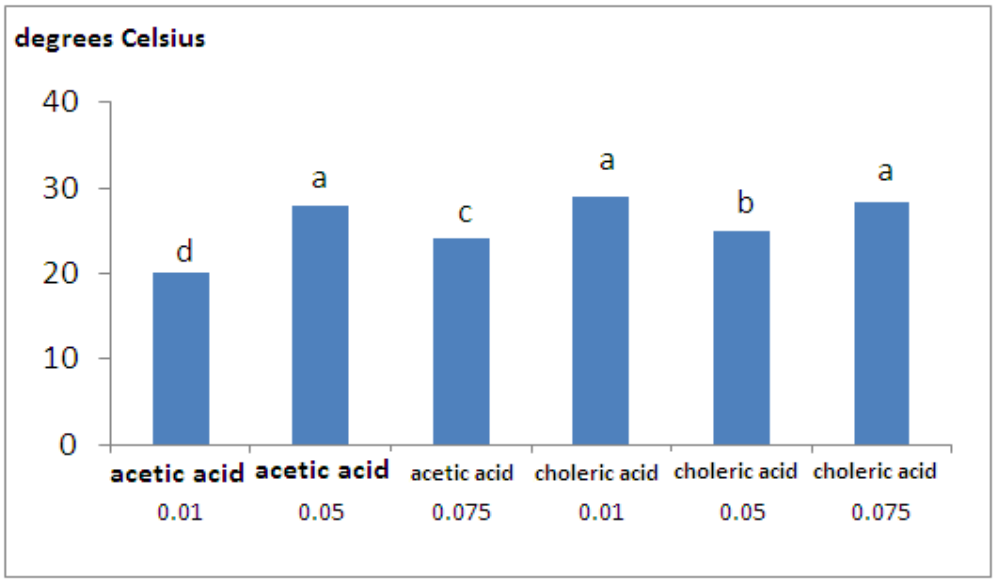


**Figure 6. Effect of acids concentration on gelatin's melting point in different treatments**

Viscosity is the second important physical and commercial characteristic of gelatin (Wasswa , Tang , and Gu, 2007). In this study, viscosity of the extracted gelatin was in the range of 1.23-9.05 mPa. According to means comparison, optimum value of viscosity in optimum point was estimated to be 9.05 mPa (fig. 8). This value has been estimated in pre-process conditions of skin with 0.01-normal sodium and 0.01-normal choleric acid. In fact, as concentration of choleric acid decreases, breakage of collagen links increases and even very fine components resulted from acidic hydrolysis act on each other, thus increasing viscosity through formation of mixtures with high molecular weight.

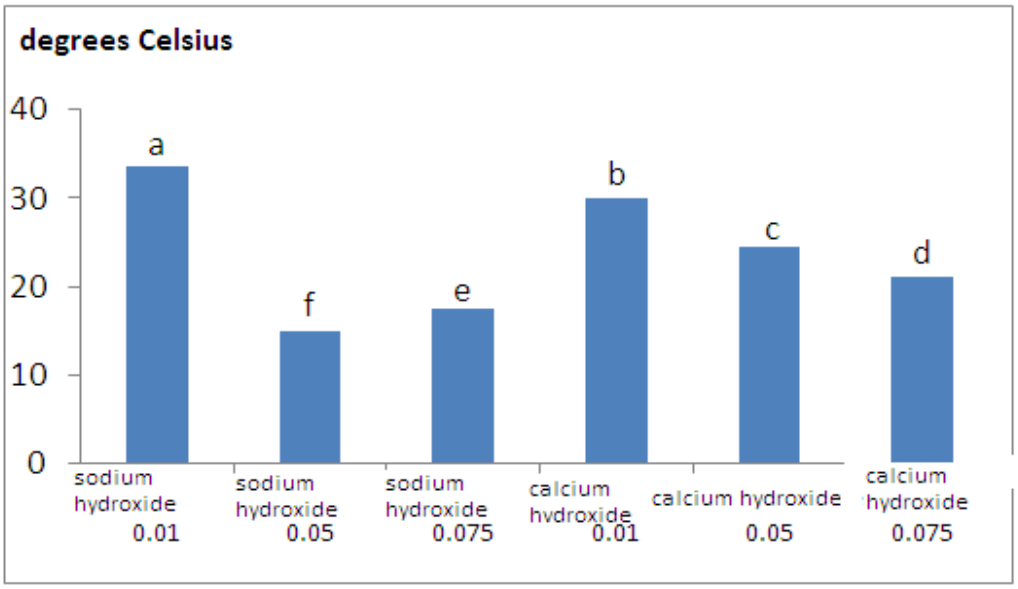


**Figure 7. Effect of alkaline concentration on gelatin's melting point in different treatments**

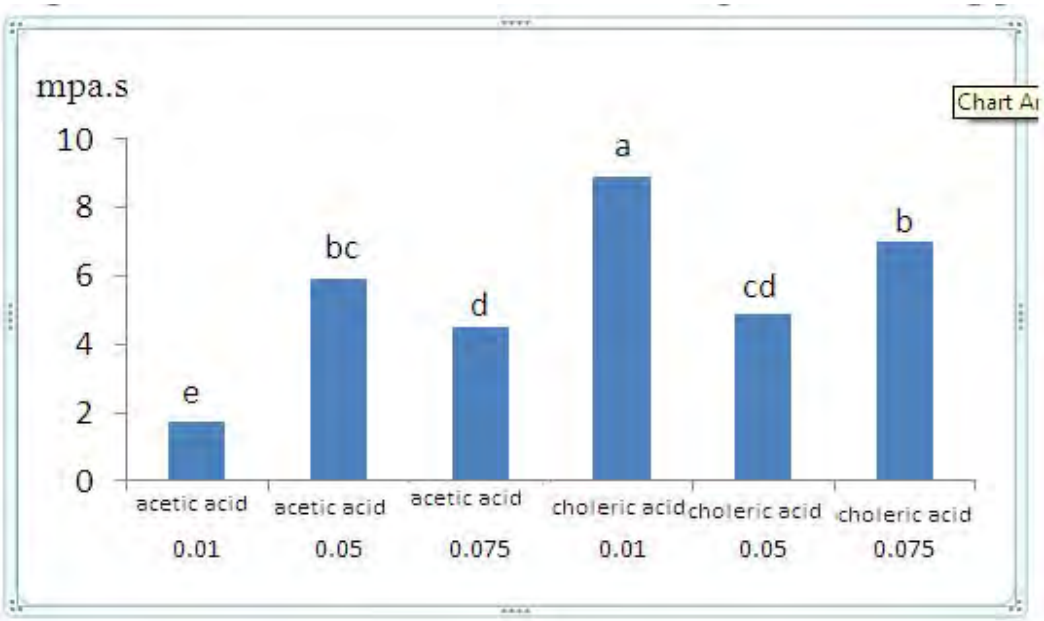


**Figure 8. Effect of acids concentration on gelatin's viscosity in different treatments**

L* (lightness), a* (redness) and b* (yellowness) of the extracted gelatin is shown in table 1. In 6.67% w-v produced gelatins, L*, b* and a* are in ranges of 21.43-41.14, 4.33-13.98, -.53 to -3.8, respectively. L* value in gelatin of the black tilapia (93.32); red tilapia (92.3) and rainbow trout (55.64) (Shahiri Tabarestani et al, 2011) had higher lightness in comparison with optimum conditions of beluga in this study. However, beluga's gelatin in current study, in terms of yellow color (b*) in optimum value (11.21) was yellower than rainbow trout (5.7), red tilapia (3.09) and paler than croaker (13.65). Gelatin's color depends on initial material's type and the number of stages but it has no effect on gelatin's functional characteristics. If we want to produce a film from gelatin or use it in a transparent drink, dark color has a negative characteristic.

Subunits constituting gelatin samples were observed via SDS-PAGE method and it was shown that generally, different pre-process conditions had significant effect on molecular weight distribution and α and β protein chains with different percentages. A chain value in gelatin samples, depending on pre-process conditions, was in the range of 10.6-66.9. According to table 2, value of α chain was estimated to be 66.9, estimated in skin pre-process conditions with 0.01-normal sodium hydroxide, 0.01-normal choleric hydroxide. High value of α chain, in lower choleric acid concentrations, relates to role of acid used in preventing further destruction of peptide structure and higher stability of peptide connections. However, in lower acetic acid concentrations, due to the existence of ion impurities in collagen, chains' ability to connect and form molecules with high molecular weight decreases.

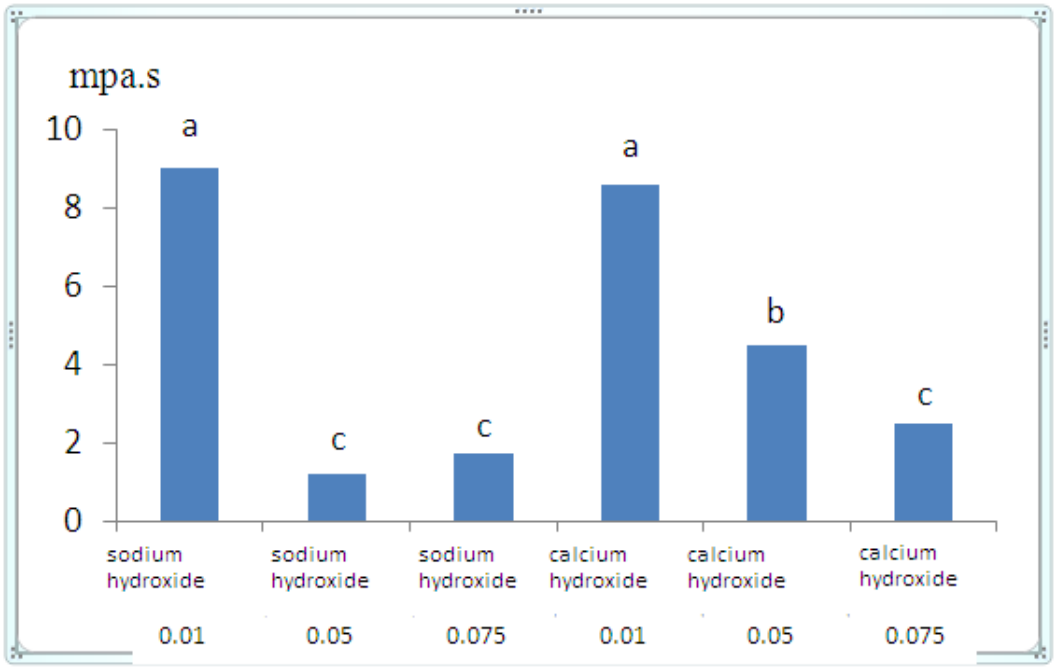


**Figure 9. Effect of alkaline concentration on gelatin's viscosity in different treatments**

High number of β chains has a considerable effect in complete reversal of collagen to collagen's natural shape and gel strength. Amount of β chains in different pre-process conditions of this study, ranged over 1.86-28.78 and concentration, type of alkali and acid had significant effects on increasing it. According to table 2, optimality value of β chain was estimated to be 28.78. This has been estimated under pre-process conditions of skin with 0.01-normal choleric acid and 0.01-normal sodium hydroxide. Hydrolysis in gelatin is a combined effect of decomposition of peptide bonds and extra-molecular lateral joints of peptide chains. Since collagen's initial structure is fixed,

difference in lateral inter-molecular joints can have a special importance in molecular weight composition of produced gelatins. In current study, β chain's value (28.78) under optimum condition in beluga's gelatin was lower than sol gelatin (42%) and megrim (38%) (Arnesen and Gildberg, 2007), and higher than young nile's skin gelatin (17.8%) (Muyonga et al, 2004).

**Table 1. Effect of acids and bases type and concentration on different characteristics of gelatine color**

| Different treatments | gelatin color | | |
|---|---|---|---|
| | a* | b* | L* |
| 0.01 acetic acid | -3.8 | 7.34 | 37.61 |
| 0.05 acetic acid | -1.11 | 4.74 | 26.22 |
| 0.075 acetic acid | -1.79 | 8.53 | 32.01 |
| 0.01 choleric acid | -0.53 | 5.66 | 24.82 |
| 0.05 choleric acid | -1.72 | 9.31 | 21.43 |
| 0.01 sodium hydroxide | -2.93 | 10.03 | 32.06 |
| 0.05 sodium hydroxide | -1.23 | 4.58 | 28.07 |
| 0.075 sodium hydroxide | -0.94 | 5.83 | 27.42 |
| 0.01 calcium hydroxide | -3.33 | 10.08 | 31.78 |
| 0.05 calcium hydroxide | -1.98 | 6.79 | 35.65 |
| 0.075 calcium hydroxide | -3.17 | 12.77 | 40.52 |

**Table 2. Effect of acids and bases type and concentration on α and β chain values**

| Treatments | α chain value | β chain value |
|---|---|---|
| 0.01-normal acetic acid | 20.24 | 14.33 |
| 0.05-normal acetic acid | 25.77 | 21.39 |
| 0.075-normal acetic acid | 30.02 | 21.44 |
| 0.01-normal choleric acid | 61.16 | 28.78 |
| 0.05-normal choleric acid | 52.44 | 24.4 |
| 0.01-normal sodium hydroxide | 66.9 | 20.63 |
| 0.05-normal sodium hydroxide | 22.47 | 1.86 |
| 0.075-normal sodium hydroxide | 10.6 | 1.86 |
| 0.01-normal calcium hydroxide | 60.85 | 25.82 |
| 0.05-normal calcium hydroxide | 21.58 | 1.86 |
| 0.075-normal calcium hydroxide | 22.77 | 1.86 |

**Conclusion**

Given the conducted study, it could be concluded that sodium and 0.01-normal choleric acid has had the biggest significant effect on increased viscosity, melting point, and gel strength. Also 0.01-normal soda and 0.05-normal choleric acid had the biggest significant effect on extraction efficiency. Furthermore, 0.075-normal calcium hydroxide and 0.01-normal choleric acid had the most significant effect on lightness of gelatin's color. In synchronous optimization of qualitative parameters, using pre-treatment solution with soda concentration of 0.01-normal and 0.01-normal choleric acid, beluga gelatin with acceptable efficiency and desired physical and chemical properties is produced. Beluga's gelatin forms gel in room temperature and for the same reason, its use is appropriate in products that are supposed to keep their jelly state in room temperature.

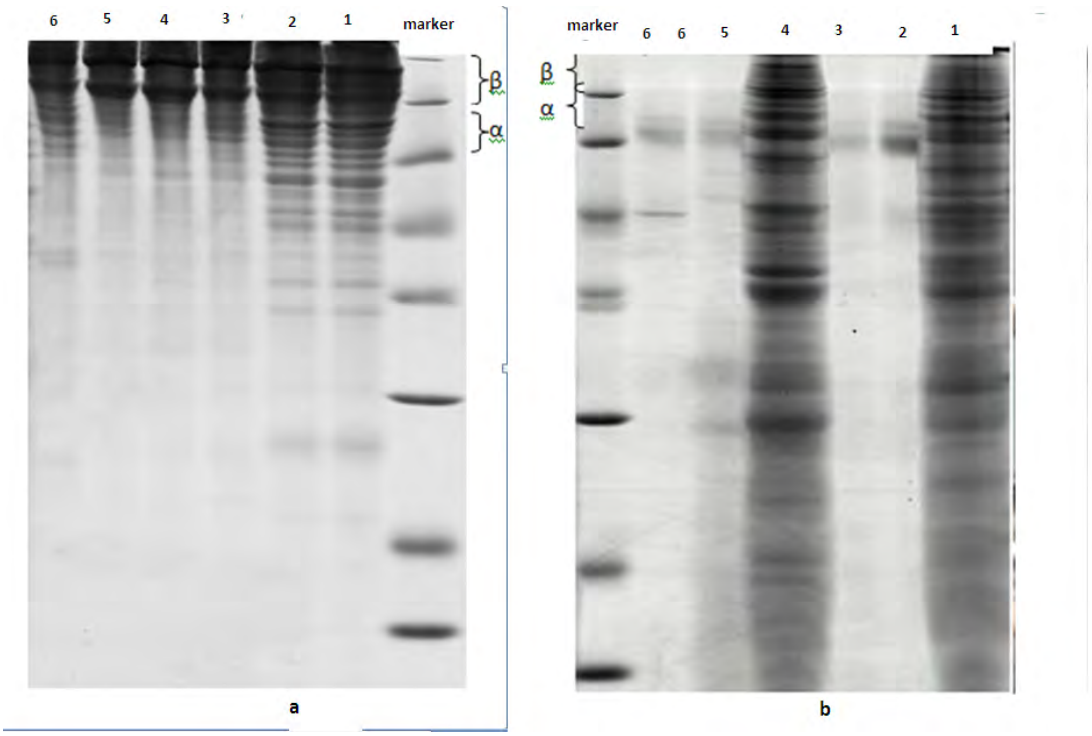


**Figure 10 electrophoresis pattern of gelatine samples under the effect of acids (a) and bases (b) type and concentration (treatment 1: 0.01-normal acetic acid, 2: 0.05-normal acetic acid, 3: 0.075-normal acetic acid, 4: 0.075-normal choleric acid, 5: 0.05-normal choleric acid, 6: 0.01-normal choleric acid)**

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