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Optimization of process condition for extraction of gelatin from red tilapia skin *(Oreochromis niloticus)* by High Pressure Processing (HPP)

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Abstract

Extraction of gelatin using traditional acid-base pretreatment method has several limitations such as time consuming and causes serious water pollution. Chemical treatment often being used as an alternative process to overcome the weaknesses of the conventional method. However, excessive chemical elements would damage the structure of the gelatin due to its high sensitivity to the acid content. High Pressure Processing (HPP) is a novel and environmental friendly method that has been suggested to assist gelatin extraction. Pressurization during pretreatment could reduce the extraction time and amount of acid used. It also has a potential in enhancing the properties of the gelatin extract and increasing the gelatin yield. In this research, One-Factor-at-Time (OFAT) and optimization study were done to determine the optimum parameters for extraction of gelatin assisted by HPP from red tilapia skin. Four parameters; applied pressure, pressure holding time, ratio of acid to skin and extraction time have been selected for the OFAT design and concentration of the gelatin extract and percentage of yield gelatin were evaluated. From OFAT, optimum technical parameters for response surface optimization design were 250 MPa pressure, 7.5 ml of acid to 1 g of skin and 12 hours extraction time. Pressure holding time was fixed for 10 min. FCCCD has been used for optimization study. Results from the data shows that the optimum conditions for gelatin extraction from red tilapia skin were 250 MPa for pressure, 10 min of pressure holding time, 7.5 ml of acid for 1 g of skin and 12 hours of extraction time while the maximum concentration and yield were 19.51 mg/ml and 32.04% (320.4 mg/g), respectively. These findings proved that HPP could increase the concentration and the yield of the gelatin while reducing the chemical waste and shortening the extraction process.

Introduction

Gelatin is a combination of several polypeptide chain α-chains, β-chains (two α-chains covalently cross-linked), and γ-chains (three α-chains covalently cross-linked) (Shankar *et al*., 2016). Two α-chain in collagen usually is characterized as hydroxyproline and proline (Wiley-VCH, 2017). Generally, gelatin with a high percentage of α-chain possesses higher gel strength. The properties of the gelatin depend on the source of collagen, type of collagen and extraction method (Gomez-Guillen *et al*., 2011). Gelatin extracted from fish skin is normally treated by acid solution. The treatment breaks the noncovalent bonds and disrupts secondary and tertiary structures of the proteins, thus resulting in adequate swelling and solubilisation of collagen. The subsequent heat treatment cleaves the hydrogen and covalent bonds and unfolds the triple-helix, resulting in helix-to-coil transition and conversion of collagen to soluble gelatin (Shankar *et al.*, 2016). Production of fish gelatin covers only 1 percent of gelatin produced worldwide (Gudipati, 2013).

There are several disadvantages of traditional acid base extraction such as chemical pollution, long processing time and large consumption of hydropower resources (Zhang *et al*., 2011; Liqing *et al*., 2012; Liqing *et al.*, 2014). Chemical treatment often used to overcome these shortcomings but excessive alkaline/acid treatment could break the intermolecular and intramolecular chain of gelatin, which will affect the amino acid content and diminish the properties of the gelatin extract (Zhang *et al.*, 2016).

Application of High Pressure Processing

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(HPP) in gelatin extraction process is gaining interest nowadays. Pressurization induces the protein denaturation by destabilizing the cross-link interaction of the non-covalent bond (Angsupanich *et al*., 1999). Besides, high pressure will force more acid to permeate into the skin interiors and allow swelling of the collagen (Liqing *et al*., 2014). HPP process also could enhance the properties of the gelatin. The degree of polymerization of the protein could be increased by high pressure, which increases the relative molecular mass fraction of the gelatin, and also increases the gel characteristics of the product (Zhang *et al*., 2011).

Until recently, there are limited number of studies on gelatin extraction assisted by HPP. The purpose of this study was to determine the optimum parameter values for gelatin extraction from fish skin assisted by HPP. The One-Factor-at-Time (OFAT) test and Response Surface Optimization (RSM) test were used to establish the process and kinetic model for gelatin extraction. The effect of applied pressure, pressure holding time, amount of acid and extraction time towards the concentration and yield of gelatin were assessed.

Materials and methods

Materials preparations

Red tilapia fish was bought from the supermarket in Gombak, Kuala Lumpur. The flesh and bone were removed while the fish skins were washed, cleaned and cut into square (1 cm x 1 cm) before stored at -20ºC until further use. Three solutions, 0.2% NaOH, 0.2% acetic acid (CH_2COOH) and 1.0% citric acid ($C_6H_8O_7$) were prepared and kept at 4ºC for at least overnight. All the chemicals used were analytical grade.

Gelatin using conventional method

Fish skin was thawed and cleaned. Gelatin extraction procedure was carried out according to Grossman and Bergman (1992) with slight modification. During pre-treatment process, skins were soaked in NaOH, acetic acid and citric acid, respectively. Each soaking period lasted for 40 minutes at 4°C with the ratio of skin to liquid was 1:14. The skins were washed thoroughly using distilled water after every soaking. Later, the treated fish skin was extracted in distilled water for 18 hours at 45ºC. Supernatant was kept for further analysis while precipitate was discarded.

Gelatin extraction assisted by HPP

Extraction of gelatin from fish skin assisted by HPP during pretreatment was done according to Gómez-Guillén *et al*. (2005) with modification. Samples were soaked in 0.2% sodium hydroxide and 0.2% sulfuric acid. Then the fish skin and 1% of citric acid were sealed in polyethylene bag before undergoing pressurization and later being extracted in distilled water at 45ºC.

Single factor experiment design

Determination of factor values were obtained from the literature (Angsupanich *et al*., 1999; Montero *et al.*, 2002; Ma and Ledward, 2004; Gómez-Guillén *et al*., 2005; Zhang *et al*., 2011; Liqing *et al*., 2012; Chang, Niu, Tang *et al*., 2013; Chang, Tang, Tang *et al*., 2013; Chang, Zhou, Yu *et al*., 2013; Liqing *et al*., 2014). The factors were gradient of applied pressure: 150, 200, 250, 300, 350 MPa; pressure holding time: 5, 10, 20, 30, 60 min; amount of acid per 1 g of skin: 1, 4.25, 7.5, 10.75, 14 and extraction Time: 6, 9, 12, 15, 18 h. OFAT test was done by changing one parameter at a time while others were fixed. Concentration of the gelatin extract and yield of gelatin were evaluated as dependent variables. The experiment were done in triplicates.

Protein concentration (Biuret test)

The biuret test was done according to Gornall *et al.* (1949). UV-Vis Spectrophotometer (Brand: Biochrom, Model: LIBRA S12) were used to obtain the absorbance reading for each concentration at 540 nm wavelength. The experiment were done in triplicates.

Yield of gelatin

Yield of gelatin extract was calculated using following equation:

Dry weight (%)= (Dry gelatin extract (g))/(Fish skin (g)) x 100

The experiment were done in triplicates.

Optimization study

Three-level Faced Centered Central Composite Design (FCCCD) from Response Surface Methodology (RSM) was selected for optimization study. Values of the parameters used in the optimization process (pressure MPa; pressure holding time, min; amount of acid for 1 g of fish skin, ml; and extraction time, hours) were based on data obtained from OFAT. Concentration of gelatin extract (mg/ml) and yield of gelatin (%) were recorded as responses. The experiment was done in triplicates. Software Design Expert 7.0.0 (State – Ease, Inc. Minneapolis,

USA) has been used to analyse the results.

Results and discussion

The concentration and yield of gelatin extracted from fish skin by using conventional method were 15.7 ±1.58 mg/ml and 25.8 ± 0.001 (%), respectively. These results will be used as controls.

One-Factor-at-Time

OFAT test was used to determine the ideal value of parameters for the optimization study. The results found that optimal parameters for optimization study were 250 MPa pressure, 10 min for pressure holding time, 7.5 ml of acid per 1 g of skin fish and 12 hours for extraction time. However, only three parameters were included in the optimization design while the pressure holding time was fixed at 10 min. This is because after 10 min, the concentration and yield of gelatin were reduced, which shows the sensitivity of the gelatin structure on the period of pressurization (Figure 1). This result was consistent with the research done by Gómez-Guillén *et al*. (2005) where the extending pressure time after 10 minutes would decrease the yield of gelatin extract. Thus, parameters for optimization study were pressure (150 – 250 MPa), ratio skin to acid (1:1 – 1:14) and extraction time (6 – 18 hours).

Optimization study

Table 1 shows optimization results for the gelatin extraction. Two responses were evaluated in this study; concentration of the gelatin extract and yield of gelatin. Examination on concentration of the gelatin extract was done using biuret method. Biuret method is a qualitative study on the presence of peptide bonds in a protein solution (Basu, 2016). The concentration of gelatin was calculated by using a standard curve prepared before performing the test ($y = 0.0586x$, $R^2 = 0.9998$). From the results, run #20 with the conditions of 150 MPa of pressure, 14 ml of acid for 1 g of sample and 18 hours of extraction time produced the poorest gelatin concentration, which is 11.78 mg/ml. In contrast, the highest result was recorded from Run #14 under the conditions of pressure 250 MPa, 7.5 ml of citric acid and 12 hours of extraction time where the concentration of gelatin was 20.63 mg/ml, shows an increment on the amount of peptide bonds compared to the conventional method. The optimum pressure and amount of acid could induce the protein denaturation by breaking and destabilizing the secondary and tertiary structure of protein (Sarupria *et al*., 2010; Basu, 2016), thus increase the number of amino acid release during

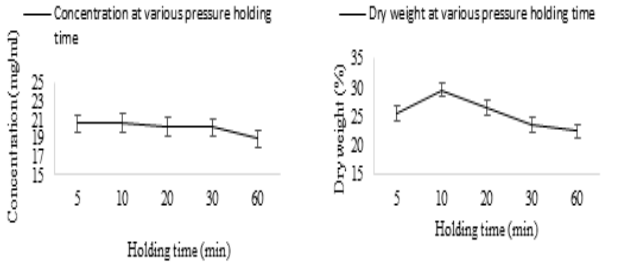

Figure 1. Effects of pressure holding time on the concentration and yield of gelation

thermal extraction. Besides, the study displays the reduction of extraction time from 18 hours to 12 hours. This finding is in parallel with the research done by Gómez-Guillén *et al*. (2005) where the extraction time could be reduced with the support from high pressure during pre-treatment.

The pressure and amount of acid usage influence the concentration of the gelatin in this study. The extract of gelatin assisted by HPP has less quantity of protein content compared to the conventional method when the amount of acid used was 1 ml per 1 g of skin (Run #5, #12, #13, #17 and #18), regardless of the pressure and the extraction time. However, findings from Liqing *et al.* (2014, 2012) show that gelatin extracted from pig skin assisted by HPP could reduce the usage of acid during pre-treatment up to a ratio of 1 g of skin to 1 ml of acid, which is inconsistence with the results obtained in this paper. This is due to the difference on the source of gelatin extract and the variety in extraction method (Mariod and Adam, 2013). Thus, extraction of fish gelatin needs more amount of acid compared to mammalian gelatin extraction. The lowest result obtained at treatment under 150 MPa, with the ratio of skin to acid is 1:14. This can be seen from run #9 and #20. Besides, results show that prolonged extraction time shows the decrement of gelatin concentration from 12.69 mg/ml to 11.78 mg/ml after increasing the time from 6 hours to 18 hours. Gelatin extract with the treatment of 350 MPa, 1:14 of ratio skin to acid and 18 hours extraction time possessed similar concentration as optimum conditions (20 mg/ml) but their process conditions do not present the advantages of HPP method since it still uses high amount of acid and at normal extraction time.

Measuring the yield of gelatin was done by dividing the dry weight of gelatin extract to their initial skin weight. Yield of gelatin for test #14 was 32% or 320 mg/g, which marks the highest result obtained from the study, with the parameters of pressure 250 MPa, 7.5 ml of citric acid and 12 hours of extraction time. Traditional method for fish gelatin extraction required 40 min for acid soaking activity

Table 1. Actual and predicted data of the Faced Centred Central Composite Design (FCCCD) of factors with concentration of gelatin and yield of gelatin as a responses

Run order	Pressure (MPa) X_1	Amount of acid (ml) for 1g of skin, X_2	Extraction time (Hours) X_3	Concentration of gelatin (mg/ml)			Yield of gelatin (%)		
				Actual	Predicted	Residue	Actual	Predicted	Residue
1	250	7.5	12	19.99	19.49	0.505	30.25	30.38	-0.130
2	250	7.5	12	18.26	19.49	-1.234	30.11	30.38	-0.270
3	250	7.5	12	20.12	19.49	0.630	30.53	30.38	0.150
4	250	7.5	18	17.08	17.92	-0.834	31.51	30.80	0.716
5	250	1	12	15.12	15.78	-0.666	23.95	24.63	-0.677
6	350	14	18	20.17	19.58	0.596	22.10	22.09	0.012
7	350	7.5	12	19.98	21.55	-1.569	27.30	27.72	-0.426
8	250	14	12	15.96	16.71	-0.755	29.18	28.86	0.320
9	150	14	6	12.69	12.59	0.105	31.85	31.51	0.336
10	250	7.5	12	20.40	19.49	0.908	29.93	30.38	-0.450
11	250	7.5	12	20.38	19.49	0.891	30.14	30.38	-0.240
12	350	1	6	13.66	13.15	0.512	21.80	21.09	0.709
13	150	1	18	15.19	15.04	0.145	25.50	25.36	0.138
14	250	7.5	12	20.63	19.49	1.141	32.04	30.38	1.655
15	150	7.5	12	18.98	18.83	0.148	29.15	29.08	0.068
16	250	7.5	6	16.75	17.34	-0.587	29.38	30.46	-1.074
17	350	1	18	15.20	14.95	0.250	26.02	26.26	-0.247
18	150	1	6	15.11	15.35	-0.241	18.95	18.87	0.077
19	350	14	6	18.32	18.11	0.210	27.85	27.90	-0.049
20	150	14	18	11.78	11.94	-0.157	26.40	27.02	-0.620

during pre-treatment (Grossman and Bergman, 1992). The purposes of acid soaking in pre-treatment are to allow mild acid to penetrate into the skin to interrupt the non-covalent bond of the gelatin structure and to obtain a sufficient swelling in order to facilitate the extraction process (Kirti and Khora, 2016). However, by applying high pressure during the treatment, more acid was forced to permeate into the skin in a short time and increase the swelling activity (Zhang *et al*., 2011; Silva and Pinto, 2012). This is the reason why the swelling time has been shortened to only 10 mins. The amount of acid used also reduced to half for optimum conditions, which resulted in reduction of chemical release into the environment (Liqing *et al*., 2012). Besides, high pressure interrupts the intermolecular reaction of the non-covalent bond which later assisting in protein denaturation. Combination of acid/HPP treatment could reduce collagen degradation because the UHP treatment mainly destabilized the balance of the non-covalent interactions of collagens, whereas the short acid treatment was not sufficient to break the peptide bonds of the collagen molecules (Liqing *et al*., 2014). As a result, during thermal hydrolysis (45°C), the inter-chain cross-linkage in gelatin structure would easily be destroyed by the heat and destabilize the triple-helix through a helix-to-coil transition and results in conversion to soluble gelatin. Since the pressure increases the breakage of the non-covalent bond, warm extraction could extract more gelation and thus reduce the extraction time (Liqing *et al*., 2012). This can be seen from the results (Table 2) when only 12 hours were needed to extract 320 mg/g of gelatin by using HPP compared to 18 hours by conventional methods (258 mg/g).

Similar to concentration of gelatin, usage of small amount of acid would produce low gelatin yield (Table 1). Besides, prolonging the thermal extraction of gelatin extraction under the treatment of pressure 150 MPa and ratio 1:14 skin to acid also shows the decline in yield of gelatin from 30% (6 hours) to 26% (18 hours). The same results could be seen from run #6 and #19 when the 350 MPa pressure was applied to the process. In contrast, when the amount of acid use is low, increasing the extraction time raises the yield of gelatin. This can be seen from run #13, #18 and #12, #17.

Residual value is based on the difference between observed and predicted value. It is also classified as an error (Gao and Sherali, 2009). The lower the amount of residual, the better it is. This is because high number of residual presents the great difference

Table 2. ANOVA for Response Surface Quadratic Model for concentration and yield of gelatin

Source	Concentration of gelatin			Yield of gelatin		
	F-Value	p-value Prob > F	Remarks	F-Value	p-value Prob > F	Remarks
Model	14.92708	0.0001	Significant	41.7850741	< 0.0001	Significant
X_1	17.64922	0.0018	Significant	6.961124398	0.0248	Significant
X_2	2.062008	0.1815		67.73704905	< 0.0001	Significant
X_3	0.791541	0.3945		0.434770081	0.5246	
X_1X_2	28.53199	0.0003	Significant	25.73252125	0.0005	Significant
X_1X_3	2.138052	0.1744		1.310993185	0.2789	
X_2X_3	0.056045	0.8176		91.22567398	< 0.0001	Significant
X_1^2	1.29762	0.2812		16.2492876	0.0024	Significant
X_2^2	27.59105	0.0004	Significant	54.99812479	< 0.0001	Significant
X_3^2	9.107577	0.0129	Significant	0.252960349	0.6259	
Lack of Fit	1.792669	0.2687	not significant	1.184834815	0.4284	not significant
Std. Dev.			1.023136			0.813131201
Mean			17.28938			27.69678736
C.V. %			5.917716			2.935832198
PRESS			43.6653			38.74376107
R-Squared			0.93			0.97
Adj R-Squared			0.86837			0.950785574
Pred R-Squared			0.711017			0.848218324
Adeq Precision			13.29094			21.98529771

between the predicted and the observed values; and the large diversion of values show the deficiency of the design. From the Table 2, average error for both responses were less than two. This clarifies the approval of the design (Kiew and Mat Don, 2012).

ANOVA analysis

Analysis of Variance (ANOVA) is a practice used to examine the effect of qualitative factors on quantitative results. ANOVA tests the effect of independent variables on a dependent variable (Christensen, 2016). It is ssential to test the significance and the adequacy of the model design. Table 2 displayed the ANOVA analysis for both responses. The most significant data required from the ANOVA results were the F-ratio. The F-ratio is equivalent to the Mean Square (variation) between the models divided by the Mean Square (residual) of the group. The model F-ratio of 14.93 for gelatin concentration and 41.79 for yield of gelatin imply models are significant. The probability of exceeding the observed F-ratio assuming no significant differences among the means (p-value) indicates that there is only a 0.01% probability that a Model F-ratio this large could occur due to noise for both responses. According to Zhang *et al.* (2010), smaller amount of P value indicates the significance of the independent variables upon the response (dependent variables). For concentration of gelatin, parameters X_1, X_1X_2, X_2X_2 and X_3X_3 has significant effects on the response ($p<0.05$) while others have less impact on the results ($p>0.05$). On the other hand, X_1, X_2, X_1X_2, X_2X_3, X_1X_1 and X_2X_2 are significant in determining the yield of the gelatin. Last but not least, the lack-of fit of the model must be insignificant ($p>0.05$) because the model must be in fit position. There were 26.87% and 42.84% chance that a "Lack of Fit F-value" this large could occur for concentration and yield of gelatin, respectively.

The regression coefficient, R^2 is an important factor that determines the relationship between response variables and parameters variables. The R^2 approaching 1 expresses that both factors and responses fit each other. From Table 2, 93% and 97% of actual value fit with the predicted values for concentration and yield of gelatin, respectively. The coefficient of variance (CV) indicates the percentage ratio of standard deviation to the mean, where the risks are common-sized and related. High value of CV will lower the reliability of the experiments. Concentration of gelatin generates 5.9% of CV while only 2.9% is reported by the yield of gelatin. These lower values indicate a greater liability of

the experiments performed. The "Pred R-Squared" of concentration of gelatin is 0.7110, which is in reasonable agreement with the "Adj R-Squared" of 0.8684. for yield of gelatin, the "Pred R-Squared" is also in agreement with "Adj R-Squared" with the value of 0.8482 and 0.9508, respectively. Lack of fit, P-value and F-value have been used to determine the adequacy of the model (Zhang *et al*., 2010). Value with more than 4 is desirable for the model. Since the ratio for both indicates an adequate signal (Table 2), the model can be used to navigate the design space.

The application of RSM based on the parameter estimate, an empirical relationship between responses and tested variable in coded units through the regression equations below for concentration of gelatin and percentage of gelatin yield.

Concentration of gelatin (mg/ml) = 15.62866 - 0.054413 x pressure + 0.50531 x ratio + 1.08548 x extraction time + 2.97263E-003 x pressure x ratio + 8.81549E-004 x pressure x extraction time - 2.19580E-003 x ratio x extraction time + 7.02633E-005 x pressure^2 - 0.076709 x ratio^2 - 0.051726 x extraction time^2

Yield of gelatin (%) = 0.8975 + 0.1155 x pressure + 3.02237 x ratio + 0.529 x extraction time - 0.0022 x pressure x ratio - 0.0005 x pressure x extraction time - 0.0704 x ratio x extraction time -0.0002 x pressure^2 -0.08607 x ratio^2 + 0.0069 x extraction time^2

Response surface methodology plays a key role in identifying the optimum values of the independent variables efficiently, under which dependent variables could achieve a maximum response (Montgomery, 2012). It occurs between two parameters while the other is fixed at central value (Ratnasari *et al*., 2014). Figure 2 (a) and (b) both illustrate a plot in convex form where the maximum concentration and yield of gelatin could be seen. Optimum parameters for a higher gelatin concentration (19.51 mg/ml) were pressure at 250 MPa, a liquid to solid ratio of 7.5 mg/ml, and the extraction time of 12 hours. Meanwhile optimal conditions for a higher gelatin yield (308.46 mg/) were pressure 225 MPa, a liquid to solid ratio of 10 mg/ml, and the extraction time of 12 hours.

Effects of amount of acid on the pH of gelatin extract

The pH value for gelatin extract for all runs were illustrated in Figure 3. pH results for all runs were within the standard of edible gelatin, which are 3.5 – 5.5 for Type A gelatin (GMIA, 2012). The highest pH recorded by run number 5 (5.09 ± 0.323) while run 19 possessed the lowest pH, which is 4.04 ± 0.029.

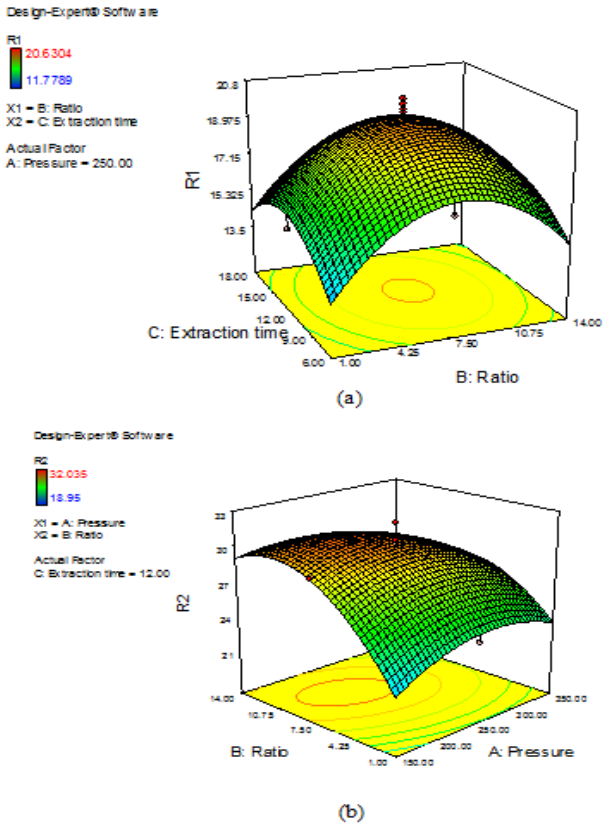

Figure 2. 3D surface for interaction between (a) extraction time and amount of acid on the concentration of gelatin extract and (b) between pressure and amount of acid on the yield of gelatin

Results shown that the amount of acid used in the experiment has a significant impact on the pH of the gelatin. In this study, three value ratio of skin and acid have been used which are 1:1 (lowest point), 1:7.5 (centre point) and 1:14 (highest point). Run with the lowest point produced less acidic gelatin extract, between 4.7 and 5 pH. It can be seen from Run #5, #12, #13, #17 and #18. On the other hand, pH of gelatin extract for ratio skin and acid at 1:7.5 were within the range of 4.4 and 4.7, as shown in the runs #1, #2, #3, #4, #7, #10, #11, #14, #15 and #16. Last but not least, experiments with the highest amount of acid used (14 ml for every 1 g of skin) produced more acidic extract solution, between 4 and 4.4 pH. This can be seen from runs #6, #8, #9, #19 and #20.

Variation of extraction times also leads to a different pH value. Longer hydrolysis period produced the less acidic gelatin extract compared to the shorter time (Figure 3). For example, runs #13 and #18, even though the pressure and ratio for both tests are the same (pressure 150 MPa, ratio skin to acid 1:1), pH for gelatin solution after 18 hours hydrolysis is less acidic compared to 6 hours extraction, which were 4.96 ± 0.153 and 4.76 ± 0.161 respectively.

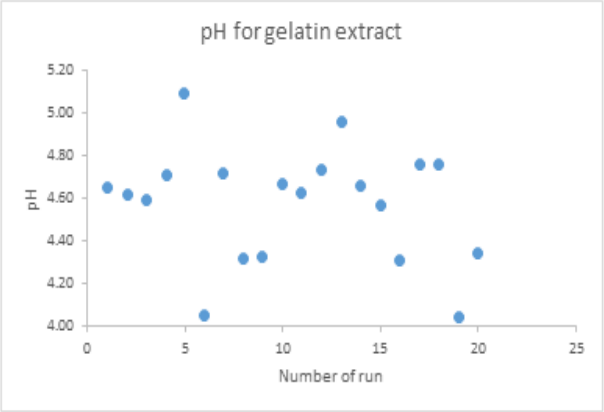

Figure 3. pH of gelatin extract for every run

Same patterns were recorded for runs 12 and 17 where there were slightly different in pH value. The difference has been highlighted more based on centre point results. Runs 1, 2, 3, 4, 10, 11, 14 and 16 have the same pressure and ratio skin:acid (250 MPa and 1:7.5, respectively). Sample from run number 16 has been soaked in hot water for 6 hours, made more acidic gelatin solution (4.31±0.232). In contrast, skin which has been hydrolysed for 12 hours in water bath at 45°C (runs 1, 2, 3, 10, 11, 14) have the pH of 4.6 and less acidic gelatin extract were recorded for run 4, where the water extraction time was 18 hours (4.70 ± 0.064). Based on the results above, the acid used during pre-treatment and extraction time has an impact on the pH of the gelatin extract. These findings are consistent with the study done by (Kiew and Mat Don, 2012) where pH of the gelatin extract was highly dependent on the acid used.

Conclusion

In light with the optimization result, it can be concluded that HPP could reduce the operation time and the amount of acid used in the extraction method. These outcomes could overcome long operational time and water pollution issues. The process and kinetic models were established by response surface test. The models are basically in agreement with the test values. They can be applied to predict the concentration and the yield of gelatin that have been extracted by HPP.

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