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APPENDICES

Appendix A

Analytical Methods

A.1 Moisture content (AOAC, 2000)

Equipments

1. Hot air oven (Model 600, Memmert, oxford, Connecticut, USA)
2. Aluminum dish
3. Desiccator

Method

1. Weigh the empty dish which was dried in a hot air oven at 105 ^{o}C for 1 hour.
2. Weigh about 2 g of sample to the dish. Spread the sample well.
3. Place the dish containing sample in the oven. Dry for 16-18 hours or until the weigh of sample is constant. Transfer the dish to a desiccator to cool for 30 minutes.
4. Weigh the dried dish containing sample. Reweigh the dish and its dried content. Calculate the moisture content using the following formulation.

$$\text{Moisture (\%)} = \frac{(W_1 - W_2) \times 100}{W_1}$$

where: W_1 = weigh (g) of the sample before drying

W_2 = weigh (g) of the sample after drying

A.2 Protein (AOAC, 2000)

Equipments

1. Buchi digestion unit (BÜCHI K-424, BÜCHI Labortechnik AG, Flawil, Switzerland)
2. Buchi distillation unit (BÜCHI B-324, BÜCHI Labortechnik AG, Flawil, Switzerland)

Chemicals

1. Sulfuric acid
2. 0.1 N Hydrochloric acid
3. 35% w/v Sodium hydroxide
4. 4% w/v Boric acid
5. Selenium reagent mixture
6. Methyl red-methylene blue indicator

Method

1. Accurately weigh ca 2 g test sample on Whatman paper, fold, and transfer to a digestion tube.
2. Add 5 g catalyst-selenium mixture, and 20 mL of conc. H_2SO_4
3. Prepare a tube containing the above chemical except sample as blank. Let the reaction subsides and place the tube in a block digestor. Heat gently until frothing ceases. Digestor must be placed in a fume hood or be equipped with an exhaust system. Boil briskly until the mixture is clear (about 45 min).
4. Remove tubes and leave to cool for about 10 min. Do not let the precipitate form; if precipitate forms, reheat. Carefully add 50 mL of H_2O. Place NaOH and boric acid solution in the alkali tank of a steam distillation unit. Attach the digestion tube containing diluted digest to distillation unit.
5. Place 250 mL receiving flask containing mixed indicator on the receiving platform, with tube from the condenser extending below the surface of the absorbing solution. Steam distilation until 100-125 mL is collected (absorbing solution turns green from liberated NH_3).
6. Remove the receiver, wash tip of condenser, and titrate the absorbing solution with 0.1 M HCl to pink colour end point. Record the volume of acid required. Titrate the blank reagent similarly. Calculate the protein content using the following formulation.

$$\text{Protein (\%)} = \frac{(A-B) \times N \times 1.4007 \times 6.25 \text{ (or } 5.4)}{W}$$

where: A = volume (mL) of 0.1N HCl used in sample titration

B = volume (mL) of 0.1N HCl used in blank titration

N = Normality of HCl

W = weigh (g) of the sample

1.4007 = atomic weigh of nitrogen

6.25 = the protein-nitrogen conversion factor for fish skin

5.4 = the protein-nitrogen conversion factor for gelatin

A.3 Fat (AOAC, 2000)

Equipments

1. Soxhlet (Gerhardt, EV-16, königswinter, Germany)
2. Rotary vacuum evaporator (EYELA N-N series, Tokyorikakikai Co., LTD, Tokyo, Japan)
3. Thimble
4. Hot air oven
5. Whatman No. 1 paper
6. Desiccator

Chemical

1. Petroleum ether

Method

1. The bottle, lid and thimble are firstly placed in an incubator at 105°C overnight to ensure that the weight of the bottle is constant.
2. Weigh about 3 g test sample onto a paper filter and wrap.
3. Put the sample into an extraction thimble and transfer into the soxhlet apparatus.
4. Fill about 250 mL petroleum ether into the bottle and place on a heating mantle.
5. Connect the soxhlet apparatus and turn on water for cooling. Switch on the heating mantle.

6. Heat the sample for about 3-4 hours.
7. Evaporate the solvent using a vacuum evaporator.
8. Dry cup and contents for 2 hours at 105°C in a hot air oven. Cool and weigh.
9. Reweigh the bottle and its dried content. Calculate fat content using the following formulation.

$$\text{Fat (\%)} = \frac{\text{Weigh of fat} \times 100}{\text{Weigh of sample}}$$

A.4 Ash (AOAC, 2000)

Equipment

1. Muffle furnaces (Fisher scientific Model Isotemp, USA)

Method

1. The crucible and lid is firstly placed in the furnace at 550°C overnight to ensure that impurities on the surface of crucible are burn off. Cool the crucible in a desiccator for about 30 minutes.
2. Weigh the crucible and lid to 4 decimal places.
3. Weigh about 2 g sample into the crucible. Heat over low Bunsen flame with lid half covered. When fume is no longer produced, place the crucible and lid in a furnace.
4. Heat at 550 °C for 8 hours or until the ash becomes greyish-white. If not, return the crucible and lid to the furnace for further ashing. Cool down in a desiccator.
5. Weigh the ash with crucible and lid. Calculate the ash content using the following formulation.

$$\text{Ash content (\%)} = \frac{\text{Weigh of ash} \times 100}{\text{Weigh of sample}}$$

A.5 Electrophoresis (SDS-PAGE) (Laemmli, 1970; Bollag et al., 1996)

Equipment

1. Electrophoresis apparatus

Chemicals and reagent preparation

1. 10% SDS
2. 2 M Tris-HCl buffer
3. 1 M Tris-HCl buffer
4. 10% Ammonium persulfate
5. TEMED (N,N,N'N' -tetramethylenediamine)
6. β- mercaptoethanol
7. Solution A (Acrylamide stock solution: 30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide): Dissolve 29.2 g of Acrylamide and 0.8 g of bisacrylamide in distilled water and make up to 100 mL and stir until compleately dissolved.
8. Solution B (Separating gel buffer: 1.5 M Tris-HCl buffer): Mix 75 mL 2M Tris-HCl (pH 8.8) and 4 mL 10% SDS and make up to100 mL with distilled water and stored at 4 °C.
9. Solution C (Stacking gel buffer: 0.5 M Tris-HCl buffer): Mix 50 mL 1M Tris-HCl (pH 6.8) and 4 mL 10% SDS and make up to100 mL with distilled water and stored at 4 °C.
10. Electrophoresis buffer: Dissolve 3 g of Tris, 14.4 g of glycine and 1 g of SDS in distilled water and adjust the volume to 1L.
11. 5x Sample buffer: Mix 0.6 mL 1M Tris-HCl (pH 6.8), 5 mL 50% glycerol, 2 mL 10% SDS, 0.5 mL β-mercaptoethanol, 1 mL 1% bromophenol blue, and make up to 100 mL with distilled water. The prepared solution was kept in 1mL aliquots and stored at -20 °C.
12. Staining solution: Dissolve 0.2 g Coomassie blue R-250 in 45 mL methanol, 10 mL acetic acid and 45 mL distilled water.
13. Destaining solution: Mix 100 mL methanol, 100 mL acetic acid and make up to 1L with distilled water.

Method

1. Gel preparation

SDS-PAGE separating and stacking gels were prepared according to the recipe in Table A5.1.

2. Assembly of electrophoresis apparatus

2.1 Assemble the electrophoresis apparatus according to the manufacture's detailed instructions.

2.2 Combine solution A and B and distilled water in a small Erlenmeyer flask. Carefully prepare solution A because it is neurotoxin. Add ammonium persulfate and TEMED and mix well. Work rapidly at this point because polymerization will be under way.

2.3 Introduce solution into gel sandwich using a pipette. This minimizes the possibility of air bubbles becoming trapped within the gel. The amount of separating gel solution should be 1.5 from top of front plat. Gentle layer of water about 1-5 mm on top of the separating gel in order to keep the gel surface flat. Allow gel to polymerize (30-60 minutes)

3. Pouring the stacking gel

3.1 Pour off completely the layer of water.

3.2 Prepare a 4% stacking gel solution to tickle into the center of the sandwich along an edge of one of the spacers.

3.3 Transfer comb into the layer of stacking gel solution by placing one corner of the comb into the gel and slowly lowering the other corner in. Be sure no bubbles are trapped on ends of teeth. Allow the stacking gel solution to polymerize 30 to 45 min at room temperature. Remove comb carefully.

Table A5.1 Formulation for SDS-PAGE separating and stacking gels.

Solution	7.5% separating gel	4% stacking gel
Solution A	5 mL	1.33 mL
Solution B	5 mL	-
Solution C	-	2.5 mL
Distilled water	10 mL	6.17 mL
10% Ammonium persulfate	100 µL	50 µL
TEMED	10 µL	10 µL
Total volume	20 mL	10 mL

4. Preparing and loading samples

4.1 Dissolve gelatin is at 4 mg/mL in 5% SDS.

4.2 Combine protein sample and 5x sample buffer in an eppendorf tube. Heat at 95-100 °C for 5 min.

4.3 Introduce protein sample 20 µL/well into well with a micropipette.

5. Running the gel

5.1 Place the upper chamber over the sandwich and lock the upper buffer chamber to the sandwich. Pour electrode buffer into the inner and outer chamber, making sure that both top and bottom of gel are immersed in buffer.

5.2 Connect the power supply to the anode and the cathode of the gel apparatus and run at 20 mA.

5.3 The dye front should migrate to 3-5 mm from the bottom of the gel. Turn off the power supply.

6. Staining and destaining gel

6.1 Stain gel in a small amount of Coomassie stain. Agitate slowly for 20-30 min.

6.2 Pour off the staining solution and pour a solution of destaining solution into the gel. Agitate slowly for about 15 min. Replace with fresh solution until the gel is clear except for the protein bands.

Appendix B

The properties of gelatin solution and nanofibers in both solvents

Table B1 The effect of acetic acid concentration on the gelatin solution properties and average gelatin nanofiber diameter.

Acetic acid concentration (% v/v)	Viscosity (cP)	Conductivity (μS/cm)	pH	Fiber diameter (nm)
10	345±23	2426±21	3.16±0.03	179±29
20	336±2	2207±40	2.81±0.07	174±23
30	401±6	1923±25	2.57±0.07	208±29
40	473±6	1764±18	2.40±0.09	233±45
50	522±11	1508±30	2.18±0.05	245±37
60	591±27	1249±22	1.97±0.07	277±32
70	699±23	1013±11	1.78±0.05	282±26
80	827±19	769±13	1.45±0.05	481±29
90	994±14	555±15	1.36±0.04	805±96
100	1079±24	268±21	1.21±0.04	2095±289

Table B2 The effect of formic acid concentration on the gelatin solution properties and average gelatin nanofiber diameter.

Formic acid concentration (% v/v)	Viscosity (cP)	Conductivity (μS/cm)	pH	Fiber diameter (nm)
10	236±24	5887±277	1.84±0.04	143±24
20	246±7	6577±244	1.38±0.07	119±18
30	251±6	7040±314	1.05±0.05	129±22
40	259±10	6760±368	0.65±0.05	134±16
50	274±13	6263±343	-	112±14
60	315±16	5707±198	-	118±17
70	357±20	4923±348	-	124±18
80	478±22	4270±341	-	137±21
90	606±15	4817±209	-	202±27
100	939±15	3103±216	-	225±28

Note: - means pH could not be measured.

Table B3 The effect of gelatin concentration on the solution properties and average gelatin nanofiber diameter when 40% v/v acetic acid was used as the solvent.

Gelatin concentration (% w/v)	Viscosity (cP)	Conductivity (μS/cm)	pH	Fiber diameter (nm)
5	39±5	1108±67	1.93±0.09	-
8	99±17	1258±50	2.13±0.05	-
11	215±27	1456±53	2.26±0.04	-
14	366±41	1601±85	2.36±0.05	161±39
17	687±38	1717±60	2.46±0.04	207±48
20	1100±24	1822±59	2.54±0.04	320±46
23	2028±75	1929±74	2.65±0.05	398±55
26	2726±90	1967±70	2.72±0.07	539±132
29	4546±108	2010±81	2.84±0.03	761±77

Note: - means no fiber was produced.

Table B4 The effect of gelatin concentration on the solution properties and average gelatin nanofiber diameter when 80% v/v formic acid was used as the solvent.

Gelatin concentration (% w/v)	Viscosity (cP)	Conductivity (μS/cm)	Fiber diameter (nm)
5	81±9	2070±70	-
8	134±31	2440±36	-
11	276±38	2640±98	-
14	479±20	3047±87	109±18
17	687±36	3160±46	177±28
20	1326±86	3303±86	238±37
23	1515±37	3450±56	262±37
26	2529±48	3613±65	284±33
29	3705±46	3657±71	302±38

Note: - means no fiber was produced and pH could not be measured.

Table B5 Mechanical properties of gelatin nanofiber mat.

Sample	Thickness (μm)	Tensile strength (Mpa)	Young's modulus (Mpa)	Elongation (%)
Acetic acid				
Gelatin 17%	128.33 ± 15.18	3.85 ± 0.27	131.46 ± 34.82	19.13 ± 7.86
Gelatin 17% (cross-link)	71±3.61	9.39 ± 0.74	478.19 ± 54.75	27.79 ± 6.93
Gelatin 20%	132.33 ± 12.58	3.95 ± 0.15	118.04 ± 7.59	37.64 ± 16.80
Gelatin 20% (cross-link)	77.33 ± 11.15	10.61 ± 0.65	523.20 ± 44.44	22.94 ± 3.56
Gelatin 23%	122 ± 9.17	2.39 ± 0.31	471.81± 111.85	28.34 ± 5.88
Gelatin 23%(cross-link)	69.33 ±10.07	6.01 ±0.24	570.01± 68.92	45.72 ± 17.07
Formic acid				
Gelatin 20%	134 ± 2.65	2.60 ±0.47	134.08 ± 19.63	3.25 ± 0.23
Gelatin 20% (cross-link)	92 ± 13.23	5.50 ± 1.17	536.42 ± 10.54	3.44 ± 1.04
Gelatin 23%	124.67 ± 11.02	4.18 ± 0.71	194.42 ± 13.52	3.98 ± 1.23
Gelatin 23% (cross-link)	87.67 ± 3.51	4.88 ± 0.92	366.43 ± 8.34	3.30 ± 0.61
Gelatin 26%	137.67 ± 15.70	6.79 ± 0.25	392.45 ± 44.43	2.75 ± 0.45
Gelatin 26%(cross-link)	75 ± 1.73	12.40 ± 1.33	880.08 ± 74.33	3.92 ± 1.98

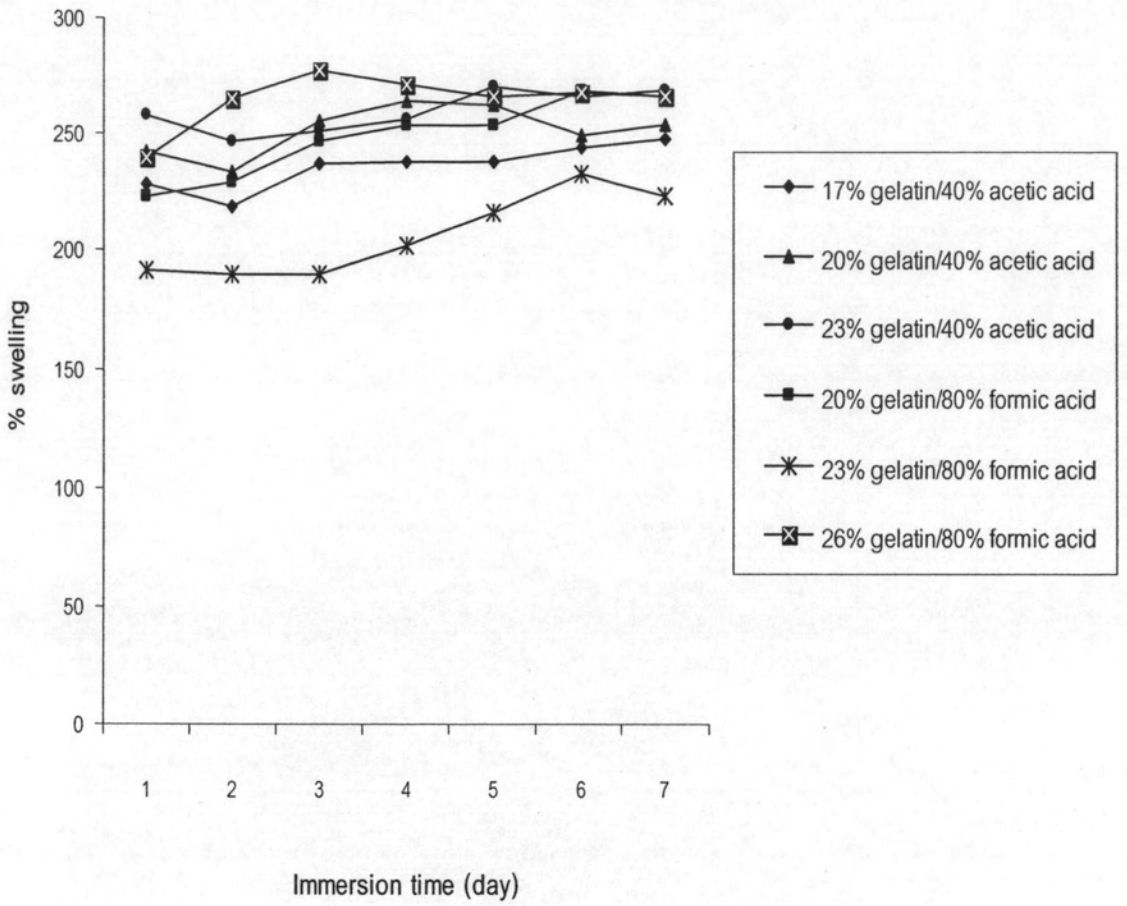

Figure B1 Swelling of the gelatin nanofiber mats after the immersion in distilled water.

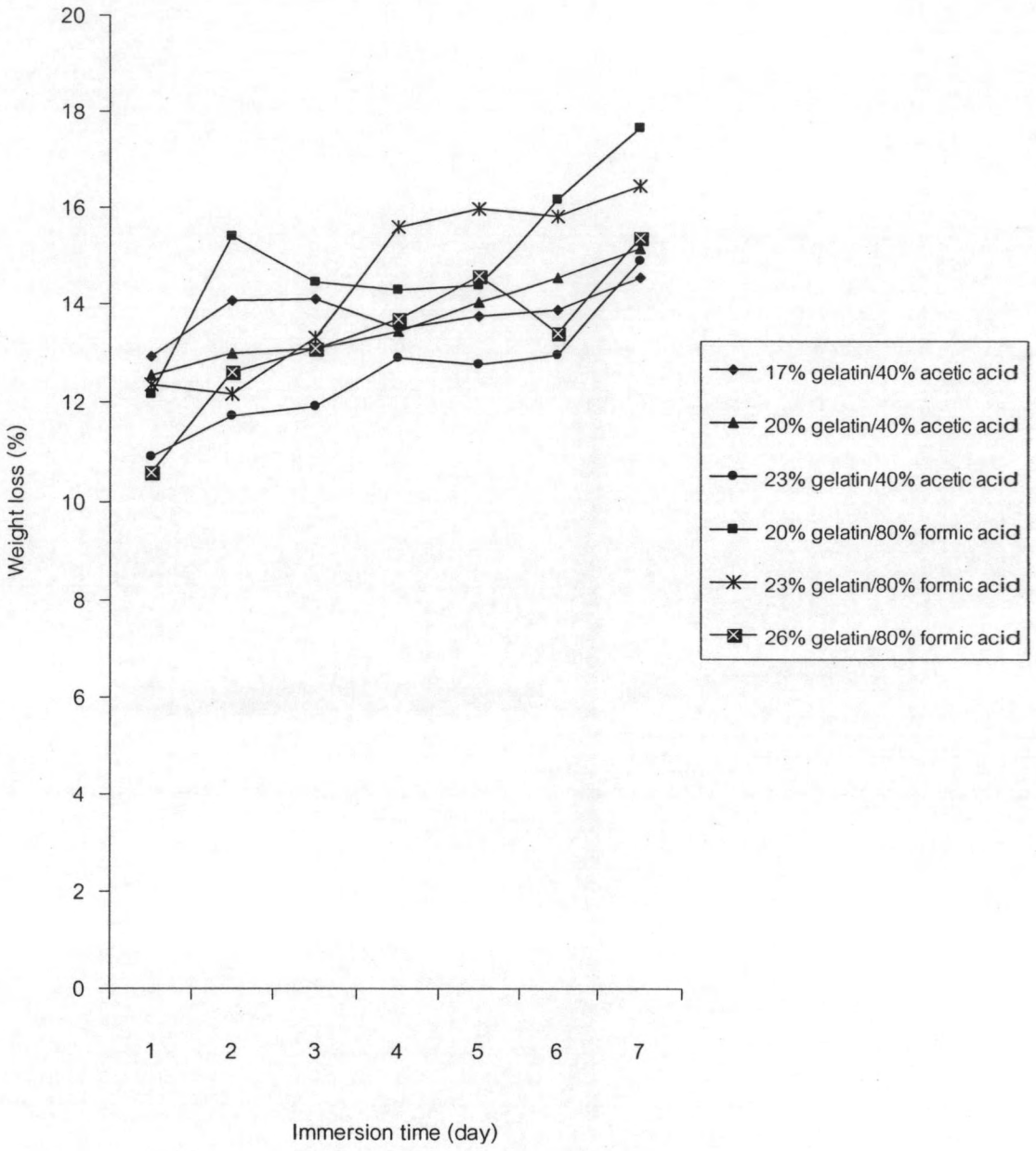

Figure B2 Weight loss of the gelatin nanofiber mats after the immersion in distilled water.

VITAE

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