7. ANNEXES

7.1. Gel preparation protocol

1) Turn on the Static Water Bath at 90°C (Reserve it for at least 2 hours)

2) Weight egg protein and water
   - 1L Beaker → 68.75 g egg / 431.25 g water
   - 2L Beaker → 137.5 g egg / 862.5 g water

   [13.75% egg in water]

3) Add egg protein slowly into water in a stirring plate at ~350 rpm to avoid too much foam formation
   - If coagulation occurs, stir cross-current with a spatula

   → Wait for 2h or until it is well dissolved ←

4) ADJUST pH [3 / 5 / 7.5]

5) Strain mixture into plastic container (to get rid of foam)
   - 1 container for 500ml gel
   - 2 containers for 1L gel

6) Label and cover container with foil

7) Place container into water bath

   → Wait for 1h ←

8) Go for ice!

9) Place container in ice (~10 min)

10) Place container in the fridge (to digest it the following day)

11) Cut the gel into perfect cubes (½ x ½ inch)
    - Use the sweet potato cutter blade
    - Use Alex's cutting board to cut borders (½ inch mark)

Note: Prepare as much gel sample as you will be putting into digestion at once on the following day

7.2. Simulated saliva and gastric juice preparation

7.2.1 Simulated saliva preparation procedure

For 500 mL of simulated saliva:

1) Add 200 mL of Milli Q water to a 600 mL beaker. Add a stir bar and place it on a stir plate.

2) Weigh 0.5 g of mucin and add it very slowly to stirring water.

3) Weigh out 1.05 g $NaHCO_3$, 0.058 g NaCl, and 0.0745 g of KCl. Add to the solution.

4) Wait for about 20 minutes for the mucin to dissolve. Cover and label the beaker during this time.

5) Pour the solution into a 500 mL volumetric flask.

6) Rinse out any remaining solution from the beaker into the flask. Add water up to the line.

7) Cover the top of the flask securely with parafilm and mix carefully.

8) Pour the solution back into the 600 mL beaker and adjust the pH to 7 using 0.1 M HCl or NaOH.

7.2.2 Simulated gastric juice preparation procedure

For 1 L of simulated gastric juice (GJ):

1) Add approximately 600mL of MilliQ water into a 1L beaker. Add a stir bar and place it on a stir plate.

2) Add 5.3 mL of 3 M HCl using the 10 mL Eppendorf pipet.

3) Weigh out 1.5 g of mucin and add it very slowly to the stirred water.

4) Weigh out 8.78 g of NaCl and SLOWLY add the salt to the stirred water.

5) Wait for around 20 minutes for the mucin to dissolve. Cover and label the beaker during this time.

6) Add the contents of the beaker to a 1000 mL volumetric flask

7) Rinse out any remaining solution from the beaker into the 1000 mL volumetric flask. Continue to add water to the line on the flask.

8) Cover the top of the flask securely with parafilm and mix carefully.

9) Add the contents of the 1000 mL volumetric flask to a labelled flask with a cap. Store in the fridge.

7.3. In vitro digestion protocol

1) Measure pH of Gastric Juice (1.8) and Saliva (7) and correct it if necessary using 1 M HCl or NaOH.

2) Heat up Gastric Juice and Saliva in water bath (37°C - 20 min)

3) Weight beakers with samples
    - Label beakers with digestion time points (30, 60, 90, 120, 180, 240 min)
    - Use 12 egg cubes in each beaker

4) Calculate how much Saliva and Gastric Juice I need
    - 0.2 ml Salvia / g sample
    - 6 ml GJ / g sample

5) Calculate how much enzymes I need to add to saliva and Gastric Juice
    - Saliva: 1.18 g a-amylase / L solution
    - GJ: 2000 units pepsin / L solution

6) Prepare the volume of saliva and the GJ needed for digestion

7) Add enzymes → At this point time starts!!

8) Add Saliva to sample

    → Wait 30 seconds ←

9) Add Gastric Juice to sample

10) Place beaker in the Shaking Water Bath (37 C - 100rpm)

11) Record the time you placed the beaker in the Shaking Water Bath.

    → After digestion ←

12) Strain solids with a kitchen sieve for subsequent analysis
    - Free amino groups quantification
    - Acidity titrations
    - Moisture content

13) Measure liquids
    - pH
    - Brix (in duplicate)

14) Clean up: Pour the Gastric Juice into the Gastric Juice container under the sink.

Note: pH-controlled samples are adjusted at 5, 15, 30, 60, 120 and 180 minutes after their first placement in the shaking water bath with 1M HCl. The pH before and after the adjustment and the volume of acid used have to be recorded.

7.4. Analyses procedure

7.4.1. OPA method protocol

1) Get ready in the fume hood:
   - Sodium Tetraborate → Solid Chemical Shelf
   - SDS → Solid Chemical Shelf
   - Glycine → Solid Chemical Shelf
   - Tetraborate Extraction Buffer → Buffer cabinet
   - OPA → Fridge
   - Methanol → Flammables cabinet
   - 2-mercaptoethanol → Flammables cabinet

2) Prepare the buffers and standard solutions:

   - **1* Sodium Tetraborate Solution (0.1M)**
     - 19.05 g Sodium Tetraborate (Na2B4O7)
     - 400ml Milli Q water

     ↳ Heat the solution a little bit to solubilize it and let it cool down afterwards

     ↳ Fill up to 500 mL with Milli Q water

     ↳ Adjust pH at 9.3

   - **2* SDS 20%**
     - 20 g SDS
     - 50 mL of Milli Q water

     ↳ Heat the solution a little bit to solubilize it and let it cool down afterwards

     ↳ Fill up to 100 mL with Milli Q water

   - **3* Tetraborate Extraction Buffer (0.0125M) (SDS 2%)**
     - 4.77 g of Sodium Tetraborate Solution (1*)
     - 10 mL SDS 20% (2*)
     - 800 mL of Milli Q water

     ↳ Fill up to 1 L with Milli Q water

     ↳ Adjust pH at 9

   - **4* Standard Solution**
     - 100 mg Glycine
     - 100 ml Tetraborate Extraction Buffer (3*)

     ↳ Mix it in a stirring plate in a 150ml beaker

4) Prepare the OPA and no-OPA solutions:

- Solution without OPA:
    - 1.25 mL of SDS 20% (2*)
    - 100 µL de 2-Mercaptoethanol
    - 1 mL Methanol

  ↳ Use a volumetric flask of 50 mL → Keep this flask labelled as no-OPA for future experiments

  ↳ Make up to 50 mL with Na-tetraborate solution (1*)

- Solution with OPA:
    - 1.25 mL of SDS 20% (2*)
    - 100 µL de 2-Mercaptoethanol
    - 1 mL Methanol-OPA *(see below)*

  ↳ Use a volumetric flask of 50 mL → Keep this flask labelled as OPA for future experiments

  ↳ Make up to 50 mL with Na-tetraborate solution (1*)

  → It's very important to use the same OPA flask every time to avoid contamination ←

- Methanol-OPA:
    - 40 mg de OPA (*o*-phthaldialdehyde)
    - 1 mL Methanol

  ↳ Weight OPA directly in the Eppendorf tube (to avoid loss)

  ↳ Vortex the tube until the solution is completely homogenized

Note: These solutions have to be prepared right before the experiment.

5) Prepare the standards for the standard curve as it is given in the *Figure 14*:

- Glycine stands for standard solution (4*)
- Extraction buffer stands for Tetraborate Extraction Buffer (3*)

Note: These solutions have to be prepared every time the experiment is performed.

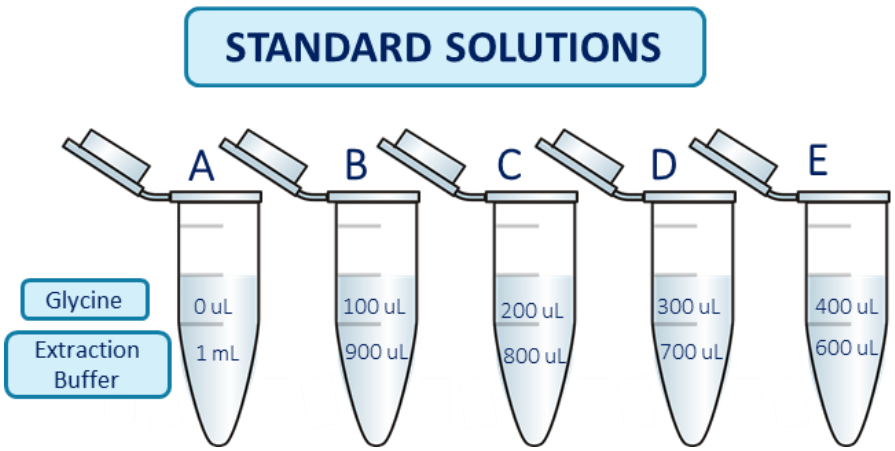

Figure 14. Graphic representation of standard solutions. Each Eppendorf tube has a different concentration of glycine with a known amount of free amino groups.

6) Read the absorbance at 340 nm of two empty microplates (OPA and no-OPA). Copy the absorbance chart in an excel spreadsheet.

7) Inject three replicates of 20 µL of all the samples in both microplates.

8) Inject 200 µL of no-OPA solution in the wells of one microplate. Watch out for bubbles as they interfere in the reading. This reaction in not time-sensitive.

9) Read the absorbance at 340 nm of no-OPA microplate. Copy the absorbance chart next to the no-OPA blank reading in the excel spreadsheet.

→ Prepare the chronometer ←

10) Inject 200 µL of OPA solution in the wells of the other microplate. Watch out for bubbles they interfere in the reading. This reaction is time-sensitive and the microplate must be read 4 minutes after the addition of the OPA solution.

11) Read the absorbance at 340 nm of OPA microplate. Copy the absorbance chart next to the OPA blank reading in the excel spreadsheet.

12) Plot the amount of free NH_2 (µg/mL) vs. absorbance (OPA – no OPA) to determine the standard curve slope and intercept to use for further calculations.

7.4.2. Acidity titrations protocols

1) Calibrate pH meter.
2) Prepare stirring plate and burette and 0.05M NaOH.
3) Add a stir bar in the sample and place it on a stir plate.
4) Measure and register initial pH.
5) Start pouring NaOH until the pH 8.2
6) Register volume spent of NaOH to reach that pH.

7.4.3. Moisture content protocols

1) Label and dry moisture content pans in the vacuum oven. There will be two pans for each time point.
2) Weight and record the mass of the empty moisture content pan.
3) Place sample on the pan and record the mass of the pan and sample combined.
4) Place the pans in the oven at a temperature of 110°C for 24 hours.
5) After drying, record the mass of each pan with the dried sample.
6) Calculate moisture content by mass difference relative to initial mass.