

# Chapter 2

## Ultra-Sensitive, High-Resolution Liquid Chromatography Methods for the High-Throughput Quantitative Analysis of Bacterial Cell Wall Chemistry and Structure

Laura Alvarez, Sara B. Hernandez, Miguel A. de Pedro, and Felipe Cava

### Abstract

High-performance liquid chromatography (HPLC) analysis has been critical for determining the structural and chemical complexity of the cell wall. However this method is very time consuming in terms of sample preparation and chromatographic separation. Here we describe (1) optimized methods for peptidoglycan isolation from both Gram-negative and Gram-positive bacteria that dramatically reduce the sample preparation time, and (2) the application of the fast and highly efficient ultra-performance liquid chromatography (UPLC) technology to muropeptide separation and quantification. The advances in both analytical instrumentation and stationary-phase chemistry have allowed for evolved protocols which cut run time from hours (2–3 h) to minutes (10–20 min), and sample demands by at least one order of magnitude. Furthermore, development of methods based on organic solvents permits in-line mass spectrometry (MS) of the UPLC-resolved muropeptides. Application of these technologies to high-throughput analysis will expedite the better understanding of the cell wall biology.

**Key words** UPLC, HPLC, Reverse-phase liquid chromatography, Cell wall, Peptidoglycan, Muropeptide

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### 1 Introduction

The interest on the biology of bacterial cell wall peptidoglycan (PG) is rising fast since the realization that, in addition to its structural role, this unique macromolecule plays critical roles in the interactions between bacteria and their environment, including other living organisms. The bacterial cell wall is a covalently closed polymeric macromolecule which is subjected to a number of modifications related with the physiological state of the cells and the environmental conditions. The canonical monomeric subunit consists of the disaccharide pentapeptide GlcNAc-( $\beta$ 1-4)-MurNAc-L-Ala-D-Glu-( $\gamma$ )-(di-amino acid)-D-Ala-D-Ala, where meso-diaminopimelic acid and L-lysine are the more frequent di-amino acids [1]. Monomers are converted into linear polymers by means of

MurNAc-( $\beta$ 1-4)-GlcNAc glycosidic bonds, and then linear polymers are covalently linked by means of peptide bridges between the peptide moieties. The final result is a net-like macromolecule which encloses the cell body. Further metabolic activities result in a series of modifications in the chemical nature of PG subunits, and on the relative proportions of the different subunits [1]. A detailed knowledge of the subunit composition under particular conditions is often relevant to understand to what extent PG variations influence bacterial adaptation to environmental challenges, resistance to anti-bacterial agents, immune-modulatory activity, and toxin release and signaling [2, 3].

The strategy to obtain compositional and structural information of the bacterial PG is based on the insolubility of this molecule in boiling SDS, and on the availability of specific enzymes (muramidases or lysozymes) able to split the MurNAc-( $\beta$ 1-4)-GlcNAc glycosidic bonds which hold the structure together. The first property permits a straightforward way to obtain fractions highly enriched in PG and the second provides a reliable way to disassemble the PG into individual subunits. A far more formidable task is to devise sensitive and reliable methods appropriate for the resolution, identification, and quantification of the different subunits, over 40 in *Escherichia coli*. The high-performance liquid chromatography (HPLC) method, originally devised by Glauner et al. in 1988 [4], was a breakthrough which revealed a completely unforeseen complexity in PG structure, and provided a reliable and sensitive analytical tool. This method has been in use for more than 25 years essentially unchanged, in spite of the dramatic improvements in instrumentation, and HPLC column materials. However, this method suffered from three critical limitations: the requirement for inorganic buffers, which complicated identification of subunits by mass spectrometry (MS), and prevented the use of in-line MS-spectrometers; the very low sample turnover (3 days for sample preparation and ca. 3 h of HPLC run time per sample); and a requirement for relatively large sample amounts (>200  $\mu$ g PG/sample). During the last years, we have been working out new methods to circumvent these limitations trying to make the best of the new instrumentation, in particular the introduction of UPLC technology and the superb properties of new materials for reverse-phase chromatography. UPLC technology allows the use of new stationary phases with a very small particle size (in the range of 2  $\mu$ m) that withstand very high pressures, increasing resolution, speed, and sensitivity. This together with improved detectors with high sampling rates and low-volume sample injectors has led to the use of smaller sample volumes (1–10  $\mu$ L) and shorter run times (5–20 min), which are essential requisites for high-throughput analysis [5].

Here we present protocols which cut down sample preparation and run times dramatically, are MS-compatible, and require about one-tenth the amount of sample. We describe sacculi isolation from bacterial cultures, both Gram-negative and Gram-positive

peptidoglycan purification, muramidase digestion, and sample preparation for LC chromatography. We describe two LC methods for the UPLC: inorganic method, which uses phosphate buffer as mobile phase, and organic method, which uses organic solvents as mobile phase and is MS compatible. The methods described can be easily adapted for the more frequently available UPLC machines by anyone with a basic knowledge of UPLC techniques. We finally provide some general instructions on data processing.

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## 2 Materials

Prepare all solutions with fresh MilliQ water. Use only ultrapure water from a distillation or deionization unit with a resistance of 8 M $\Omega$ /cm at 25 °C and analytical grade reagents. pH is critical in most solutions used (*see Note 1*). Solutions are stored at room temperature unless otherwise indicated.

As several reagents are toxic or harmful, part of the work must be carried out in a fume hood, e.g., use of HF for Gram-positive bacteria muropeptide isolation or preparation of mobile phases. Consult the product safety information and material safety data sheets of chemicals and dispose of the products conveniently.

### 2.1 *Sacculi* Preparation

1. Phosphate buffer saline (PBS 1 $\times$ ): Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na<sub>2</sub>HPO<sub>4</sub>, and 0.24 g KH<sub>2</sub>PO<sub>4</sub> in 800 mL MilliQ water. Adjust to pH 7.4 using concentrated HCl. Adjust volume to 1 L with MilliQ water. Sterilize by autoclaving.
2. Lysis buffer: SDS 5% (w/v) in MilliQ water.
3. 12–50 mL tubes (*see Note 2*).
4. Hot plate stirrer and magnets (*see Note 3*).

### 2.2 *Gram-Negative* *Bacteria* Peptidoglycan Purification

1. Benchtop ultracentrifuge, TLA-100.3 rotor, and ultracentrifuge tubes (3 mL polycarbonate tubes).
2. MilliQ water.
3. 2 mL Eppendorf tubes.
4. 1 mg/mL Pronase E stock: Dissolve 10 mg Pronase E (EC 3.4.24.31) in 10 mL Tris-HCl 10 mM pH 7.5 NaCl 0.06% (w/v). Prepare 1 mL aliquots and store at –20 °C. Pronase E must be activated prior to digestion by incubating for 30 min–1 h at 60 °C (*see Note 4*).
5. SDS 10% (w/v) solution in MilliQ water.

### 2.3 *Gram-Positive* *Bacteria* Peptidoglycan Purification

1. Benchtop ultracentrifuge, TLA-100.3 rotor, and ultracentrifuge tubes (3 mL polypropylene tubes) (*see Note 5*).
2. MilliQ water.
3. 1.5 and 2 mL Eppendorf tubes.

4. 10–15 mL tubes (*see* **Note 6**).
5. Glass beads (diameter 0.1 mm) and vortex.
6. Lyophilizer (or SpeedVac concentrator).
7. SDS 10% (w/v) solution in MilliQ water.
8. 100 mM Tris–HCl pH 7.5: Dissolve 12.11 g Tris in 900 mL MilliQ water. Adjust to pH 7.5 using concentrated HCl. Adjust volume to 1 L with MilliQ water.
9. 10 mg/mL  $\alpha$ -Amylase stock: Dissolve 100 mg  $\alpha$ -amylase (Sigma-Aldrich) in 10 mL MilliQ water. Prepare 500  $\mu$ L aliquots and store at  $-20\text{ }^{\circ}\text{C}$ .
10. 1 M  $\text{MgSO}_4$ : Dissolve 12 g  $\text{MgSO}_4$  in 80 mL MilliQ water. Adjust volume to 100 mL with MilliQ water.
11. Nuclease mix: 100  $\mu\text{g}/\text{mL}$  DNase I (EC 3.1.21.1) and 500  $\mu\text{g}/\text{mL}$  RNase A (EC 3.1.27.5). Mix enzymes in 1 mL MilliQ water, prepare 200  $\mu\text{L}$  aliquots, and store at  $-20\text{ }^{\circ}\text{C}$ .
12. 2 mg/mL Trypsin stock: Dissolve 20 mg trypsin (EC 3.4.21.4) in 10 mL MilliQ water. Mix and prepare 1 mL aliquots. Store at  $-20\text{ }^{\circ}\text{C}$ .
13. 50 mM  $\text{CaCl}_2$ : Dissolve 555 mg  $\text{CaCl}_2$  in 80 mL MilliQ water. Adjust volume to 100 mL with MilliQ water.
14. 8 M LiCl: Dissolve 33.9 g LiCl in 80 mL MilliQ water. Adjust volume to 100 mL with MilliQ water.
15. 100 mM EDTA pH 7.0: Dissolve 2.9 g EDTA in 80 mL MilliQ water. Stir vigorously on a magnetic stirrer. Adjust pH to 7.0 with concentrated NaOH and adjust volume to 100 mL with MilliQ water.
16. Acetone.
17. Hydrofluoric acid 48% (v/v) (HF).

#### **2.4 Muramidase Digestion**

1. 1.5 mL Eppendorf tubes.
2. Digestion buffer: 50 mM Phosphate buffer pH 4.9. Dissolve 682.6 mg  $\text{NaH}_2\text{PO}_4$  and 14.3 mg  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$  in 80 mL MilliQ water and stir until completely dissolved. Adjust pH to 4.9 with diluted orthophosphoric acid (*see* **Note 7**). Adjust volume to 100 mL with MilliQ water.
3. 1 mg/mL Muramidase stock: Dissolve 10 mg Cellosyl (EC 3.2.1.17) in 10 mL digestion buffer (phosphate buffer 50 mM pH 4.9). Vortex thoroughly until completely dissolved and prepare 1 mL aliquots. Store at  $-20\text{ }^{\circ}\text{C}$ .

#### **2.5 Sample Reduction and Filtration**

1. 1.5 mL Eppendorf tubes and long glass tubes.
2. Borate buffer: Borate buffer 0.5 M pH 9.0: Dissolve 3.1 g boric acid in 80 mL MilliQ water and stir until completely

dissolved. Adjust pH to 9.0 with concentrated NaOH. Adjust volume to 100 mL with MilliQ water.

3. Freshly prepared 2 M NaBH<sub>4</sub> solution: Dissolve 76 mg NaBH<sub>4</sub> in 1 mL MilliQ water (*see Note 8*).
4. Orthophosphoric acid 25 % (v/v).
5. pH-indicator strips: pH range 5.0–10.0 and pH range 0.0–6.0.
6. 96-Well filter plate (regenerated cellulose filter, 0.2 µm pore size), multititer 96-well plates (350 µL, V-bottom (*see Note 9*), and pierceable adhesive seal (*see Note 10*).
7. Vacuum manifold and pump.

## 2.6 UPLC Separation

1. Acquity UPLC system (Waters).
2. Analytical columns: Kinetex C18 UPLC Column 1.7 µm particle size, 100 Å pore size, 150 × 2.1 mm (Phenomenex).
3. Precolumn filters or guard columns: Security guard ultra cartridges C18 for 2.1 mm ID columns and holder (Phenomenex).
4. Inorganic buffer A: 50 mM Phosphate buffer, pH 4.35. Prepare a 10× buffer A solution by dissolving 40 g NaOH in 1800 mL MilliQ water. Adjust pH to 4.35 with orthophosphoric acid (*see Note 11*). Adjust volume to 2 L with MilliQ water and add 2 mL sodium azide 2 % (w/v). Keep stock solution at room temperature. To prepare the working solution, mix 200 mL 10× buffer A with 1800 mL MilliQ water and adjust pH to 4.35 (if necessary, carefully adjust pH with orthophosphoric acid 25 % (v/v)). Filter buffer with a filter membrane (nylon or nitrocellulose, 0.45 µm pore size) and a vacuum pump.
5. Inorganic buffer B: 50 mM Phosphate buffer, pH 4.95, 15 % methanol (v/v). Prepare a 10× buffer B solution by dissolving 60 g NaOH in 1800 mL MilliQ water. Adjust pH to 4.95 with orthophosphoric acid (*see Note 12*). Adjust volume to 2 L with MilliQ water. Keep stock solution at room temperature. To prepare the working solution, mix 170 mL 10× buffer B with 1530 mL MilliQ water and adjust pH to 4.95 (if necessary, carefully adjust pH with orthophosphoric acid 25 % (v/v)). Add 300 mL HPLC-grade methanol and mix. Filter buffer as described above.
6. Organic buffer A: Formic acid 0.1 % (v/v). Dilute 2 mL HPLC-grade formic acid in 2 L MilliQ water and mix.
7. Organic buffer B: Formic acid 0.1 % (v/v), acetonitrile 40 % (v/v). Dilute 2 mL HPLC-grade formic acid and 800 mL acetonitrile in 2 L MilliQ water and mix.

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## 3 Methods

### 3.1 *Sacculi Preparation*

1. Grow cultures to the desired optical density in the appropriate culture medium for the bacteria (*see Note 13*).
2. Harvest the cells at 3000 g for 15 min and resuspend the pellet in 1.5 mL PBS 1× or its own media (*see Note 14*).
3. Transfer the sample to tubes and place a stirring magnet (*see Notes 2 and 3*). Add 1.5 mL boiling lysis solution and place tubes on a beaker with boiling water on a magnetic hot stirrer plate (*see Note 15*). Let the samples boil for 30 min–3 h (*see Note 16*). Finally, switch off the hot plate and let the lysate stir overnight (*see Note 17*).

### 3.2 *Gram-Negative Bacteria Peptidoglycan Isolation*

1. Wash sacculi by spinning down the samples for 10 min at 20 °C and 150,000 × *g* using 3 mL polycarbonate ultracentrifuge tubes (*see Note 18*). Fill the tubes with 3 mL lysate. Make sure that the rotor is properly balanced. After centrifugation, all soluble compounds will remain in the supernatant. Carefully discard it with a vacuum pump without removing the pellet (*see Note 19*). Resuspend the pellet in 900 μL MilliQ water and check for the presence of SDS (*see Note 20*). If needed, add 2 mL MilliQ water, mix, and centrifuge again. Repeat this wash step until SDS is completely removed (*see Note 21*).
2. Activate Pronase E 1 mg/mL by incubation for 30 min–1 h in a 56–60 °C water bath (*see Note 22*).
3. Transfer the resuspended pellet from Subheading 3.2, step 1 (~900 μL), to 2 mL Eppendorf tubes (*see Note 23*). Add 100 μL activated Pronase E 1 mg/mL. Incubate samples at 56–60 °C during 1 h (*see Note 24*). Stop the reaction by adding 110 μL SDS 10% (w/v) and boil for 5 min in water (*see Note 25*).
4. Let the sample cool down before transferring to the ultracentrifuge tubes. Wash Pronase E-digested sacculi by adding 2 mL MilliQ water. Mix and ultracentrifuge as described above. Carefully discard the supernatant and, after total removal of SDS, resuspend the pellet in 100 μL MilliQ water or digestion buffer (*see Note 26*).

### 3.3 *Gram-Positive Bacteria Peptidoglycan Isolation*

1. Once the samples are cooled down to room temperature, concentrate the sacculi by ultracentrifugation during 10 min at 20 °C and 150,000 × *g* using 3 mL polypropylene ultracentrifuge tubes (*see Note 18*). Fill the tubes with 3 mL lysate. Make sure that the rotor is properly balanced. After centrifugation, soluble compounds will remain in the supernatant. Carefully discard it with a vacuum pump without removing the pellet (*see Note 27*). Resuspend the pellet in 500 μL

MilliQ water and check for the presence of SDS (*see Note 20*). If needed, add 2 mL MilliQ water, mix, and centrifuge again. Repeat this wash step until SDS is completely removed (*see Note 28*).

2. Transfer the resuspended pellet from Subheading 3.3, **step 1** (~500  $\mu\text{L}$ ), to 2 mL Eppendorf tubes. Add 200 mg glass beads and break the cells vigorously vortexing at 4 °C during 15 min (*see Note 29*).
3. Leave the tubes stand for 1 min on the bench or make a short spin at  $2000\times g$  to allow the glass beads and unbroken cells to precipitate to the bottom of the tube (*see Note 30*) and carefully pipette the supernatant into an ultracentrifuge tube (*see Note 31*).
4. For maximum sample recovery, add 500  $\mu\text{L}$  MilliQ water to the glass beads and unbroken cell pellet and repeat **steps 2** and **3**. Mix the recovered sample in the ultracentrifuge tube used before (final volume of sample ~1 mL).
5. To concentrate the sacculi, add 2 mL MilliQ water, mix thoroughly, and ultracentrifuge during 10 min at 20 °C and  $150,000\times g$ .
6. Resuspend the pellet in 1 mL 100 mM Tris-HCl, and transfer it to a 15 mL tube (*see Note 6*). Add 10  $\mu\text{L}$  10 mg/mL  $\alpha$ -amylase and incubate for 2 h at 37 °C with vigorous shaking (*see Note 32*).
7. For nucleic acid degradation, add 1 mL 100 mM Tris-HCl, 40  $\mu\text{L}$  1 M  $\text{MgSO}_4$ , and 2  $\mu\text{L}$  nuclease mix. Incubate the samples for 2 h at 37 °C with shaking.
8. Treat the sample with trypsin by adding 100  $\mu\text{L}$  2 mg/mL stock solution and 50  $\mu\text{L}$  50 mM  $\text{CaCl}_2$ . Incubate for 16 h at 37 °C with magnetic stirring (*see Note 33*). Inactivate the digestion by adding 200  $\mu\text{L}$  SDS 10% (w/v) and boil the samples for 10 min.
9. Transfer the samples to ultracentrifuge tubes and centrifuge during 10 min at 20 °C and  $150,000\times g$ . Remove the supernatant and wash the insoluble material as described in Subheading 3.3, **step 1**, until total removal of the SDS.
10. Resuspend the SDS-free pellet in 1 mL 8 M LiCl and incubate for 10 min at 37 °C (*see Notes 34* and *35*). Add 2 mL MilliQ water and ultracentrifuge the sample during 10 min at 20 °C and  $150,000\times g$ . Carefully remove the supernatant.
11. Resuspend the pellet in 1 mL 100 mM EDTA and incubate for 10 min at 37 °C (*see Notes 34* and *36*). To remove EDTA, add 2 mL water and ultracentrifuge the samples during 10 min at 20 °C and  $150,000\times g$ .



12. Remove the supernatant and wash the pellet with 1 mL acetone (*see Note 37*). Add 2 mL MilliQ water and ultracentrifuge during 10 min at 20 °C and 150,000×*g*. Carefully remove the supernatant (*see Note 38*). To completely remove acetone, resuspend the pellet in 1 mL MilliQ water, increase volume up to 3 mL with MilliQ water, and ultracentrifuge again.
13. Resuspend the pellet in 500 µL–1 mL MilliQ water, transfer the sample to Eppendorf tubes, and dry them using a lyophilizer (*see Note 39*).
14. To remove the teichoic acids, resuspend the pellet in 1 mL chilled 49% HF and transfer the sample to a 10–15 mL plastic tube. Stir for 48 h at 4 °C with a magnetic stirrer (*see Note 40*).
15. Transfer the samples to ultracentrifuge tubes, add 2 mL MilliQ water, and mix. Centrifuge for 10 min at 20 °C and 150,000×*g* (*see Note 41*). Discard the supernatant in the proper waste container.
16. Resuspend the pellet in 1 mL MilliQ water, increase volume up to 3 mL with MilliQ water, and ultracentrifuge for 10 min at 20 °C and 150,000×*g*. Repeat this step once and finally resuspend the pellet in 100 µL MilliQ water or digestion buffer (*see Note 26*).

### 3.4 Muramidase Digestion

1. Transfer resuspended sacculi (~100 µL from Subheadings 3.2, step 4, or 3.3, step 16) to 1.5 mL Eppendorf tubes.
2. Add 2 µL muramidase 1 mg/mL and let the reaction work for 2–16 h at 37 °C (*see Note 42*).
3. Muropeptides are now in the soluble fraction. Boil the samples for 5 min to stop the reaction (*see Note 43*). Centrifuge for 15 min at room temperature and 20,000×*g* in a benchtop centrifuge and transfer the muropeptide-containing supernatant to 1.5 mL Eppendorf or to long glass tubes (*see Note 44*).

### 3.5 Sample Reduction and Filtration

1. Add borate buffer to the sample to adjust pH to 8.5–9.0. For a 100 µL reaction, 15–20 µL borate buffer is typically used. Check pH using indicator strips (*see Note 45*). The pH now is more alkaline to make the reduction step not very fast (*see Note 46*).
2. Add 10 µL freshly prepared NaBH<sub>4</sub> 2 M and let the sample reduce at room temperature for 20–30 min (*see Note 47*).
3. Adjust sample pH to 2.0–4.0 with orthophosphoric acid 25% (v/v) (*see Note 48*).
4. Transfer reduced samples to a 96-well filter plate (0.2 µm pore size, regenerated cellulose). Using a vacuum manifold recover the filtered samples in a 96-well multiter plate (*see Notes 49 and 50*).



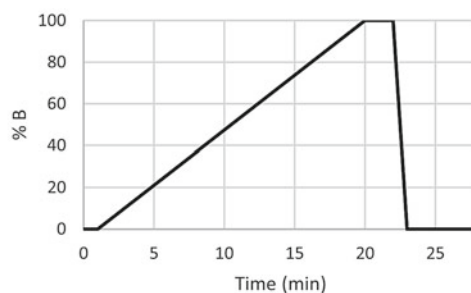
### 3.6 UPLC Inorganic Separation

1. Set the column temperature to 35 °C.
2. Prepare mobile phases and refill bottles A and B with inorganic buffer A (phosphate buffer 50 mM, pH 4.35) and inorganic buffer B (phosphate buffer 50 mM, pH 4.95, methanol 15 % (v/v)), respectively. Purge pumps and tubes according to the UPLC system instructions (*see Note 51*).
3. Equilibrate the column with inorganic buffer A, flow 0.25 mL/min, until pressure is stabilized (*see Notes 52 and 53*).
4. Using the system auto-sampler, inject 10  $\mu$ L sample (*see Note 54*).
5. Perform the LC run using the gradient described in Fig. 1 and measure absorbance at 204 nm (*see Notes 55–60*).

### 3.7 UPLC Organic Separation

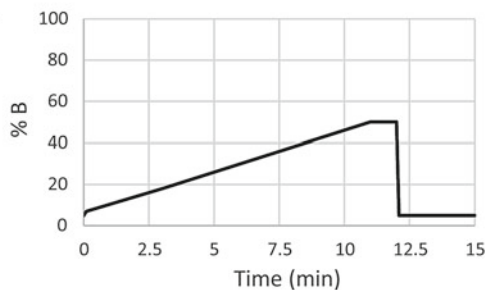
1. Set the column temperature to 45 °C.
2. Prepare mobile phases and refill bottles A and B with organic buffer A (formic acid 0.1 % (v/v)) and organic buffer B (formic acid 0.1 % (v/v), acetonitrile 40 % (v/v)). Purge pumps and tubes according to the UPLC system instructions (*see Note 51*).
3. Equilibrate the column with organic buffer A, flow 0.175 mL/min, until pressure is stabilized (*see Notes 53 and 60*).
4. Using the system auto-sampler, inject 10  $\mu$ L sample (*see Note 54*).
5. Perform the LC run using the gradient described in Fig. 2 and measure absorbance at 204 nm (*see Note 55–60*).

Time (min)	% A	% B	Comment
0	100	0	
1	100	0	
20	0	100	To wash the column
22	0	100	
23	100	0	To reequilibrate the column
28	100	0	



**Fig. 1** UPLC inorganic gradient

Time (min)	% A	% B	Comment
0	95	5	
0.1	93	7	
3	82	18	
11	50	50	To wash the column
12	50	50	
12.1	95	5	To reequilibrate the column
15	95	5	



**Fig. 2** UPLC organic gradient

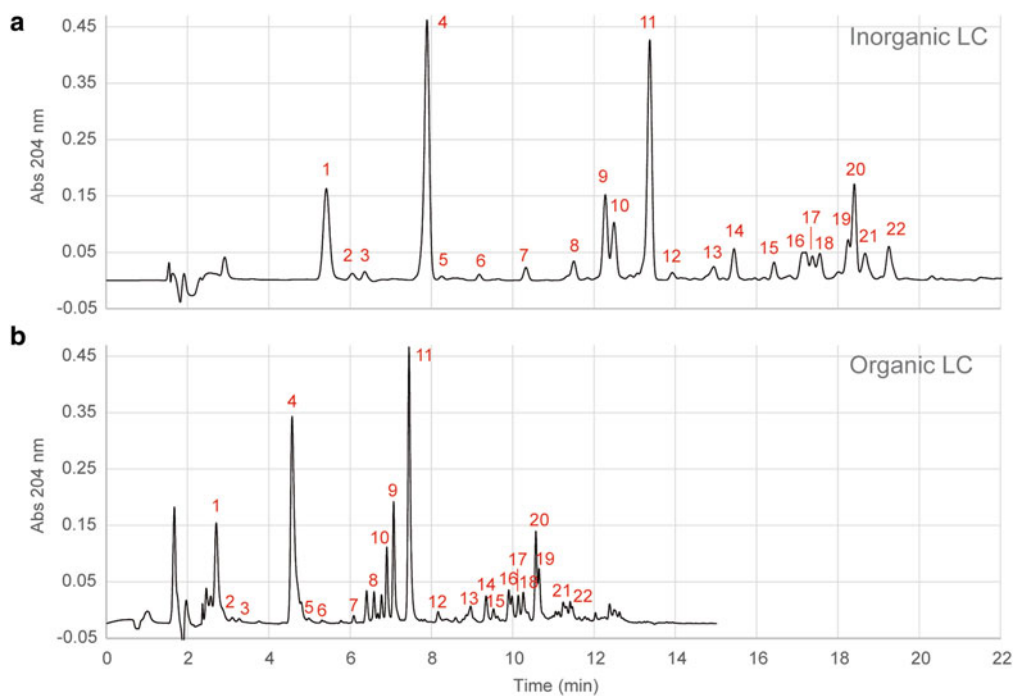
### 3.8 UPLC Data Processing

1. Extract the raw data and represent the chromatogram by plotting absorbance at 204 nm (arbitrary units) against retention time (min) (*see Note 60*).
2. Define chromatographic processing regions removing unuseful data (*see Note 61*).
3. Use appropriate software for peak alignment (*see Note 62*).
4. Calculate the area of each peak by integration using the appropriate software (e.g., UPLC manufacturer's software, MATLAB) and determine the relative abundances for each peak (*see Note 63*).
5. Determine the identity of each peak by comparison to a known reference chromatogram, e.g., Fig. 3a, b (*see Note 64*).
8. Represent the results as a muropeptide table that typically contains retention time and relative abundance for all detected muropeptides (Fig. 3c).

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## 4 Notes

1. When measuring the pH, always fix the pH electrode in a vertical position and gently stir the solution. The pH of most solutions is temperature dependent; therefore adjust at a temperature as close as possible to the temperature the buffers are going to be used.
2. Make sure that the tubes are suitable for boiling. We usually use conical centrifuge tubes (e.g., 15 mL Falcon tubes or similar).
3. Magnets should be small enough to fit in 12–50 mL tubes. We usually use 12 × 6 mm stirring bars.
4. Pronase E is a mixture of at least three caseinolytic activities and one aminopeptidase activity used for removal of peptidoglycan-bound proteins such as Braun's lipoprotein. The mixture retains activity in SDS 1% (w/v). The product can be completely inactivated by heating above 80 °C for 15–20 min.  
Other proteases as chymotrypsin (EC 3.4.21.1) and trypsin (EC 3.4.21.4) can also be used.
5. It is absolutely necessary to use polypropylene tubes or other acetone and HF-resistant material for Gram-positive peptidoglycan extraction. Otherwise, tubes will degrade and sample will be lost.
6. We usually use 15 mL conical centrifuge tubes.
7. Finely adjust pH using orthophosphoric acid 25% (v/v). Concentration of Na is critical in the inorganic system; therefore if pH goes below the indicated values by more than a few hundredths of a unit, it should not be readjusted with NaOH.



Peak	Murop.	Inorganic LC		Organic LC	
		RT (min)	%	RT (min)	%
1	M3	5.42	10.17	2.70	11.32
2	n.d.	6.06	0.58	3.10	0.24
3	M4G	6.36	0.63	3.27	0.30
4	M4G	7.89	23.63	4.56	25.20
5	M2	8.58	0.55	4.97	0.54
6	M5	9.18	0.38	5.30	0.15
7	M3-Lpp	10.33	1.00	6.08	0.50
8	D33	11.50	1.91	6.58	1.77
9*	D34	12.28	7.11	7.06	7.76
10*	D43	12.49	5.13	6.89	4.61
11	D44	13.37	19.94	7.44	20.67
12	D45	13.93	0.65	8.16	0.87
13	n.d.	14.94	1.44	8.96	1.72
14	T444	15.44	2.42	9.34	2.08
15	n.d.	16.43	1.30	9.52	0.93
16	D34N/D43N	17.17	3.45	9.93	3.77
17	n.d.	17.37	1.68	10.13	1.76
18	n.d.	17.55	2.09	10.25	2.36
19*	D44N	18.25	2.77	10.64	3.82
20*	D44N	18.40	7.24	10.56	5.42
21	D45N	18.67	2.74	11.27	2.24
22	T444N	19.25	3.20	11.43	1.97

**Fig. 3** *Vibrio cholerae* peptidoglycan profiles. Representative chromatograms from a *Vibrio cholerae* sample analyzed using the inorganic (a) and the organic (b) LC methods. (c) Relative abundance of muuropeptides. RT retention time (min). %: relative abundance. n.d. not determined. \*: muuropeptides with a shift in the retention time dependent on the LC method

8. Sodium borohydride ( $\text{NaHB}_4$ ) is a reducing agent highly soluble in water and lower alcohols. It reacts with these solvents to produce  $\text{H}_2$  in a quite violent reaction. Do not close any tube lids.
9. V-bottom multititer 96-well plates ensure maximum recovery of the sample upon injection.
10. Alternatively, samples can be individually filtered using 1 mL syringes and 4 mm syringe filters (4 mm regenerated cellulose filters, 0.2  $\mu\text{m}$  pore size) and maximum recovery vials with caps.
11. We usually add 55 mL orthophosphoric acid 85 % (v/v) and then finely adjust the pH mL by mL with orthophosphoric acid 25 % (v/v).
12. We usually add 50 mL orthophosphoric acid 85 % (v/v) and then finely adjust the pH mL by mL with orthophosphoric acid 25 % (v/v).
13. For optimal sample preparation, a minimum of  $10^{10}$  bacteria cells are required.  $10^9$  bacteria produce a small pellet after ultracentrifugation that is easily lost during the washing steps. We usually grow 10 mL cultures up to stationary phase. Larger volumes require scaling up reagents and are more time consuming, but provide better results.
14. Resuspension volume is not critical, although it is preferred to use the minimal volume possible to reduce the number of washes by ultracentrifugation.
15. When boiling SDS is added, cells will lyse immediately and the lysate will become transparent, proteins will solubilize while the sacculi remain intact. For larger sample volumes, optimal lysis is achieved by pouring the sample suspension drop by drop into an equal volume of boiling lysis solution, in tubes with stirring bars, inside a beaker of boiling water.
16. Samples need stirring during the boiling process to shear the DNA, which otherwise will interfere with the formation of a compact pellet upon centrifugation. For Gram-positive bacteria boiling with SDS can be shortened to 30 min since nuclease treatment will be performed later. Also, cell amount will affect the efficiency of the lysis, so longer boiling times are required for higher cell concentrations. Remember to add water to the beaker from time to time due to the high evaporation rate.
17. For Gram-positive bacteria, overnight stirring is not required.
18. Centrifuging at low temperatures will cause SDS precipitation.
19. The resulting pellet is transparent and can be difficult to localize, so it is advisable to mark one side of the tube and place the tube with this side up in the rotor. After centrifugation, the pellet will be located at the bottom in the opposite side of the tube.

20. Resuspend the pellet and agitate the bottom of the tube. Bubble formation is indicative of detergent presence. High sacculi concentration can also produce bubbles, but they usually disappear immediately after the agitation.
21. Usually, for  $10^{10}$  cells only two washes are required.
22. To save time, this step can be performed during the previous washing steps. Activated Pronase E is stable at 4 °C during 24 h.
23. Pronase digestion and inactivation can be performed in the ultracentrifuge tubes. However, this will shorten the lifetime of these tubes and hence disposable tubes are preferred.
24. Pronase E will remove peptidoglycan-bound proteins such as Braun's lipoprotein. Some bacteria do not require Pronase E treatment, but it is recommended for removal of other peptidoglycan-associated proteins.
25. Boiling in SDS 1% (w/v) will denature Pronase E and it will be removed from the supernatant during the next washing steps.
26. Muramidase digestion is equally effective in both solutions. This final volume is important because after the digestion the muropeptides will remain in the soluble fraction. If peak intensity after LC is too low, this volume can be decreased. However, scaling down the volumes can cause difficulties during the reduction and filtering steps.
27. Gram-positive bacteria sacculi pellets are not transparent like in Gram-negative bacteria. However, provided that the polypropylene tubes are translucent it is recommended to mark one side of the tube for helping localizing the pellet (*see Note 19*).
28. Usually, for Gram-positive bacteria at least three washes are required.
29. Cells can also be broken by sonication with glass beads as described in [6] or with the help of a FastPrep instrument as described in [7].
30. Long centrifugations lead to loss of sample due to an excess of cell precipitation. In our experience, letting the tubes stand for a few minutes gives the best results.
31. Avoid pipetting the precipitated pellet in the ultracentrifuge tube.
32.  $\alpha$ -Amylase hydrolyzes  $\alpha$  bonds of large,  $\alpha$ -linked polysaccharides. This treatment is used to remove glucose polymers from the sample. Its use depends on the composition of the peptidoglycan of each bacterial species and can be skipped sometimes.
33. Trypsin is a protease that degrades peptidoglycan-bound proteins.

34. Transferring the sample to new tubes is not required.
35. LiCl is used for extraction of peptidoglycan-associated proteins [8–10].
36. EDTA is a chelating agent that will remove the LiCl from the sample, which could interfere with the muramidase digestion.
37. Acetone removes phospholipids from the cell wall fraction and thus facilitates protein extraction [11]. Due to its corrosive nature, do not add more than 1 mL to avoid spilling and rotor damaging.
38. After the acetone wash, pellets are usually not very compact and they easily detach from the tube walls. Hence, removal of acetone is better performed using the pipette instead of decanting the tube.
39. Resuspension volume is not critical and will depend on the amount of pellet. Smaller volumes will be dried faster. Aliquoting the sample in several tubes reduces drying time. Alternatively, samples can be dried using a SpeedVac concentrator.
40. When working with HF, follow the local safety and laboratory regulations, use the flow hood, and make sure that the materials are suitable for the use of this acid. HF is pre-cooled on ice before use. Incubating the samples for less than 48 h gives bad or poor PG isolation.
41. If volumes are scaled up, do not centrifuge more than 1.5 mL HF to avoid spilling and rotor damaging. Rinse the rotor, lid, and O-ring with distilled water after use to completely remove HF and prevent corrosion.
42. After 2 h of reaction, 95 % of the sample has been digested. We usually leave the reaction overnight to ensure total digestion.
43. It is critical not to add detergent to the sample. Traces of SDS in the sample cause altered migration profiles during LC separation. Muramidase is inactivated by boiling the sample and precipitation by centrifugation.
44. For sample reduction of Gram-positive bacteria or high reaction volumes long glass tubes are preferred, since bubble formation can lead to loss of sample.
45. Do not use the indicator strip on the sample directly. In order to save sample, take 0.5  $\mu$ L and drop it on the indicator strip. Then, check the color/pH on the reference table.
46. Do not leave the sample for a long time in an alkaline pH because it leads to  $\beta$ -elimination (the disaccharide loses the peptide).
47.  $\text{NaBH}_4$  is very reactive and needs to be prepared immediately before use.  $\text{H}_2$  is produced and there will be bubble formation. Prevent gas accumulation by leaving the lids opened. Due to the constant bubble formation, it is necessary to ensure

that the right volume of  $\text{NaBH}_4$  is pipetted. Improper sample reduction will result in peaks and profiles with altered retention times. The reaction takes place during the first minutes, so it is better to add an excess of  $\text{NaBH}_4$  than to leave the reaction stand for a long time.

48. This step is critical for adequate separation of the mucopeptides by UPLC. pH higher than 5 units will result in mucopeptides eluting with altered retention times, especially when using the organic LC separation method. Add 4  $\mu\text{L}$  orthophosphoric acid 25% (v/v) to the sample and check pH using indicator strips as indicated before. Carefully add acid  $\mu\text{L}$  by  $\mu\text{L}$  to ensure that the sample has the desired pH.
49. Prior to injection, samples need to be filtered to remove impurities (*see Note 10*).
50. For long-term storage, samples are preferably stored in glass vials (minimizing solvent evaporation through pre-slit cap mats and avoiding potential leaking of contaminants from well plates). If analysis is undertaken soon after preparation, prepared samples should be kept at 4 °C (on ice or in the refrigerator) until they are transferred to the auto-sampler. If necessary, prepared samples can be stored frozen at -20 °C before analysis. However, this may lead to the formation of insoluble precipitates, which should be removed immediately before injection via centrifugation or heating the sample.
51. For mucopeptide profiling, reverse-phase (RP) columns, typically C18-bonded silicas that are able to retain and separate medium-polar and nonpolar metabolites, provide a good separation pattern. For RPLC, maximum retention of analytes is ensured by loading samples onto the column using solvents of low eluotropic strength (i.e., composed mainly or entirely of water). Elution of retained metabolites is accomplished using a gradient of increasing methanol (inorganic method) or acetonitrile (organic) content.
52. In our system, pressure oscillates between ~7300 and ~8600 psi during the inorganic run.
53. To ensure repeatability of the separations, parameters such as flow rate and column temperature need to be carefully controlled, following the manufacturer's indications. With ~12–20-min runs and a 3- to 5-min re-equilibration period, ~50 samples can be run per day. In our experience, we can run ~2000 samples on each chromatographic column before observing pressure issues or substantial degradation in peak quality.
54. The injection volume can be modified depending on the sample concentration. For concentrated samples (e.g., peptidoglycan from Gram-positive bacteria or from large starting cultures), inject less volume. If there are no peaks or absorbance is too low, increase the injection volume.



55. If the chromatograms show poor peak shapes, this is indicative of column degradation or sample overloading. To solve this problem, dilute the sample or improve sample preparation. If the problem persists, consider cleaning or replacing the column.
56. If there are no or few peaks, either the injection failed (try reinjecting the sample) or sample concentration is too low. In this case, inject larger volumes, concentrate the sample using a Speedvac concentrator, or prepare new sample.
57. Changes in the retention time can be due to improper sample pH or presence of detergent (SDS) in the sample. When sample pH > 5, it results in shifted chromatograms in the organic method but not the inorganic one (due to its buffering effect). Adjust pH with orthophosphoric acid 25% (v/v) and rerun the sample. If there is detergent in the sample, either prepare new sample increasing the amount of washing steps or wash the column after each run to get rid of the retained detergent.
58. Extra peaks appear due to poor sample reduction. Reduce the sample again or prepare new sample. Contamination with other components or short re-equilibration time between runs also contributes to the appearance of ghost peaks.
59. If peak separation is not optimal, optimize the gradients for longer run times. This can be useful for some Gram-positive bacteria peptidoglycan profiles where the amount of peaks overcomes the resolution capability of the detector.
60. In our system, pressure oscillates between ~3800 and ~4100 psi during the organic run.
61. We usually remove the solvent front at the beginning of the run (typically 1–2 min) and the final re-equilibration step (min 22 for the inorganic method and min 12 for the organic method).
62. For new or unknown samples, a reference sample can be run to facilitate peak alignment and muropeptide identification.
63. Divide the area of every peak by the total area of the chromatogram (sum of all individual areas) and multiply by 100 to calculate relative abundance in %.
64. Retention times differ depending on the gradient and UPLC system used. For confirmation of peak identity, individual peaks need to be collected and subjected to MS analysis.

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