# Detection of the bacterial quorum sensing signaling molecules *N*-acyl homoserine lactones (HSL) and *N*-acyl-homoserine (HS) with an enzyme-linked immunosorbent assay (ELISA) and via ultrahigh performance liquid chromatography coupled to mass spectrometry (UHPLC-MS)

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Running head: Detection of bacterial quorum sensing molecules with ELISA and UHPLC-MS

**Abstract**

Quick, reliable quantitative methods requiring low amounts of sample volume are needed for the detection of *N-*acyl homoserine lactones (HSL) and their degradation products *N*-acyl homoserines (HS) in order to elucidate the occurrence and dynamics of these prevalent quorum sensing molecules of Gram-negative bacteria in natural samples and laboratory model experiments. A combination of ELISA and UHPLC-MS is presented here which has proven to meet these requirements. Both methods cannot only precisely detect and quantify HSLs but also their degradation products HS and thereby enable studying signaling dynamics in Quorum sensing which have been identified to play an essential role in bacterial communication.

**Key Words**

*N*-acyl homoserine lactone, Quorum sensing, bacterial signaling, ELISA, UHPLC-MS

## Introduction

For the analysis of Quorum sensing (QS) signaling in Gram-negative bacteria it is mandatory to measure exactly what kind of signaling substances are present in a certain habitat and at what concentration. For the best known QS signaling substance, *N*-acyl homoserine lactones (HSLs), several detection methods have been developed, e.g. biosensor assays, thin layer chromatography, gas and liquid chromatography without or coupled with mass spectrometry [[1-7](#_ENREF_1)]. All these methods have certain advantages and drawbacks, but many of them have in common, that quantification especially of low amounts of autoinducers is difficult, although these low concentrations have been shown to be relevant in natural systems. Furthermore, it is often not possible to detect the degradation products of HSLs, the *N*-acyl homoserines (HSs), which are formed due to the activity of lactonases cleaving the lactone ring or due to abiotic degradation when the pH in the vicinity of the cells rises above 7. This often leads to an underestimation of produced HSLs and prevents to monitor the fate of HSLs over a longer time period. It has become more apparent in recent years, that HSL degradation is an integral part of the QS signaling process and facilitates a reset of the autoinducer system in order to respond quickly to environmental changes [[7-9](#_ENREF_7)].

As one method to quantitatively detect HSLs and their degradation product HSs, a competitive ELISA in the coating antigen format was established in our labs [[1](#_ENREF_1)]. The applied monoclonal antibody (mAb) had the advantage to bind to both HSL and HS with different affinity (quantified by the cross reactivity value, CR). By measuring each sample before and after a hydrolysis step the signal intensity of the untreated sample, consisting of an unknown ratio of HSLs and HSs, can be compared to the signal intensity of the hydrolyzed sample consisting only of HSs. By this the individual amounts of HSL and HS in each sample can be calculated. For the ELISA a microtiter plate is coated with a BSA conjugated antigen (HSL-BSA). Sample and standard HSL or HS are incubated with a defined amount of monoclonal antibody (mAb) specific for the HSL/HS present in the sample. This mixture is then transferred to the antigen coated plates, where only those mAbs can bind to the antigens on the plate, which are not saturated with the acyl-HSL/HS molecules present in the sample/standard. These mAbs are then detected by a peroxidase coupled secondary antibody, which facilitates a colorimetric quantification. Thus, the stronger the color intensity, the less analyte was present in the sample/standard solution. The described method provides a fast, sensitive, and cost-efficient way to detect acyl-HSLs/HSs in biological samples, omitting complex sample preparation. Another advantage is the low amount of sample required (< 1 mL). One critical point is however, that the method is prone to attenuating matrix effects.

To become independent from the sample matrix and to precisely quantify even low concentrations of acyl-HSL/HS molecules within small amounts of cell cultures, a UHPLC-MS method was established [[7](#_ENREF_7)]. The sample preparation is adapted for culture supernatants and intact cellular compartments separately to reach the most efficient extraction effect for HSLs/HSs. A relative long (10 min) reversed-phase separation is used to clearly isolate different side chain lengths of HSLs/HSs, which is confirmed by the MS detection in positive ionization mode.

## Materials

All chemicals should be at least of analytical grade and LC-MS grade solvents needed for chromatographic separations must be filtered through 0.2 µm membrane filters. Aquatic solutions are prepared using ultrapure water with a conductivity of 18 MΩ-cm and a total organic carbon lower than 5 ppb at 25 °C. Read the material safety data sheets of the used chemicals and diligently follow all regulations regarding personal protection, exposure controls and disposal considerations.

### ELISA

Unless indicated otherwise store all reagents at 4°C.

1. Primary antibody and coating antigen: For the preparation of suitable AHL specific antibodies and BSA conjugated coating antigens please refer to [[10](#_ENREF_10),[1](#_ENREF_1)]. The primary antibody has to be selected according to the size of the *N*-acyl side chain (long with more than 6 C atoms or short with 6 or less) and the substitution at the C3 atom.
2. Secondary antibody: 5 µg/mL of Goat-anti-rat-POD (0.4 mg/mL stock concentration) in 40 mM PBST pH 7.6
3. Coating antigen solution: 0.2 μg/mL HSL-BSA antigen in 50 mM carbonate buffer, pH 9.6
4. Wash solution PBST: 4 mM PBS, 0.05% v/v Tween-20 in H2Oultrapure pH 7.2
5. Blocking solution: 1 g Casein in 100 mL 40 mM PBS, pH 7.6. Prepare fresh every time and stir until needed.
6. HSL standard stock solution: 1 mg/mL solution in acetonitrile of the HSL in question
7. HS standard stock solution: Hydrolyze HSL solution with 1M NaOH as described in the method section for the samples to obtain the 1 mg/mL HS standard stock solution.
8. Standard dilution series: This depends largely on the sensitivity of the antibody in use and has to be optimized for each application. The values given here are just an example. The antibodies characterized by [[1](#_ENREF_1)] were more sensitive to HS, therefore the concentration of the HS standard could be 5 times lower. HSL and HS stock solutions are diluted preferably in the medium the bacteria were cultivated (or an appropriate buffer) according to the following two tables:

**Table 1:** HSL-Standards

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| µL buffer | 950 | 960 | 500 | 960 | 500 | 960 | 500 | 800 | 900 | 900 |
| µL pipetted from previous dilution | 50  | 240 | 500 | 240 | 500 | 240 | 500 | 200 | 100 |  |
| Conc. [ng/mL] | 50000 | 10000 | 5000 | 1000 | 500 | 100 | 50 | 10 | 1 | 0 |

**Table 2:** HS-Standards

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| µL buffer | 990 | 1080 | 500 | 960 | 500 | 960 | 900 | 900 | 900 | 900 |
| µL pipetted from previous dilution | 10  | 120 | 500 | 240 | 500 | 240 | 100 | 100 | 100 |  |
| Conc. [ng/mL] | 10000 | 1000 | 500 | 100 | 50 | 10 | 1 | 0,1 | 0,01 | 0 |

1. Hydrolysis: 1M NaOH, 1M HCl
2. Substrate: 0.4 mM 1-Step Ultra TMB-ELISA (Thermo), 1.3 mM H2O2 in 100mM sodium acetate buffer, pH 5.5
3. Stop solution: 2M H2SO4
4. Plates: MaxiSorp plate (NUNC), U-bottom microplate low binding (NUNC)

### Hybrid magnetic microparticles-solid phase extraction (HMP-SPE) of culture supernatants

1. Hybrid magnetic microparticles (HMP): Weigh 0.27 g iron(III)-chloride in a glass vial and add 0.70 g sodium acetate. Dissolve the mixture in 8 mL ethylene glycol (*see* **Note 1**). Add 100 mg of the copolymer Oasis HLB (*see* **Note 2**) to the solution and homogenize it by stirring. To form HMP place the glass vial in a PTFE-lined stainless steel digestion vessel and keep it in an oven for 12 h at 200 °C (*see* **Note 3**). Separate HMP and reaction solvent by use of an external magnet and wash them at least 5 times with 5 mL purified water. Repeat washing steps with methanol. Finally, keep HMP at 60 °C until dryness [[7](#_ENREF_7)].
2. Methanol, LC-MS grade
3. Purified deionized water
4. Elution solvent: Fill 7.5 mL of 3-propanol in a glass vial and add 2.5 mL hexane (*see* **Note 4**)
5. Redissolving solvent: Fill 9.0 mL purified deionized water in a glass vial and add 1.0 mL acetonitrile (*see* **Note 4**).
6. HSL standard solutions: Prepare standard solutions in a concentration range of 0.1-10.0 µM using cell culture medium or acetonitrile for dilution (*see* **Note 5**).

### HSL/HS extraction of cell pellets

1. Extraction solvent: Prepare 100 mL of anhydrous ethyl acetate and acidify the solution with 10 µL glacial acetic acid.
2. Re-dissolving solvent: Prepare a 100 mL mixture of 50 %v/v anhydrous ethyl acetate and 50%v/v acetonitrile (*see* **Note 4**).

### UHPLC-ESI-QToF-MS analysis

1. UHPLC Column: Use a Waters Acquity BEH C18 column with the dimensions 1.0 x 150 mm and a particle size of 1.7 µm or a column with similar separation properties.
2. Eluant A: Prepare a solution of purified deionized water with 10 % v/v acetonitrile and 0.1 % v/v formic acid.
3. Eluant B: 100 % acetonitrile.

## Methods

### Sample preparation

In principle liquid samples from any given habitat can be analyzed. However, the matrix can lead to a considerable inhibition of the assay and needs to be thoroughly tested. Especially rich bacterial culture medium like nutrient broth (NB) is likely to cause a significant matrix effect and should be avoided.

* + - 1. Separation of cellular compartments and cell culture supernatants should be achieved by (ultra-)centrifugation at 4 °C (*see* **Note 6**).
			2. Subsequently, the supernatants should be filtered through 0.22 μm nitrocellulose membranes and frozen at −80 °C for further extraction and analysis (*see* **Note 7**).

### ELISA

The given amounts are calculated for 3 plates. Measure all standards and samples at least in triplicates and take mean. Mix all components in glass beakers and then pour in specifically labeled plastic reservoirs for multi-pipettes. If used for one component only, these plastic reservoirs can be reused after rinsing with tab water. Plates should be covered with PCR foil (reusable if clean) during every incubation step. It is advisable to process not more than three plates in parallel and always finish one complete step with one plate before repeating the step with the next one.

1. Coating: Prepare coating antigen solution by diluting 1 µL stock solution of HSL‐BSA in 34.5 mL 50 mM carbonate buffer. Add 100 μL of antigen solution per well into a Nunc Maxi Sorp microtiter plate and incubate overnight at 4°C without shaking.
2. Preparations: On the next day put all buffers out of the refrigerator to equilibrate to room temperature. If you are using an automated washer (see next step) attach vacuum and buffer bottles, and perform a washing cycle with an empty 96 well plate.
3. General washing procedure: Wash the coated plate three times with 200 μL 4 mM PBST buffer per well. This can be done manually with a multichannel pipette or with an automated washer, e.g. with an automated microtiter plate washer from Bio-Tek Instruments (Bad Friedrichshall, Germany). After washing empty the plate completely by tapping it with open wells down on a paper towel. Do not let the plate dry out after the washing process, but immediately proceed to next step.
4. Blocking step: Prepare 1% blocking solution by adding 1 g of Casein to 100 mL of 40 mM PBS, pH 7.6 and stir until needed. Add 300 µL blocking solution per well. Place the plate on a plate incubator at room temperature shaking at 500 rpm until needed (not longer than 3-4 h).
5. Standard preparation: Prepare HSL and HS standards in bacterial culture medium (or appropriate buffer) according to the list under point 8 in the Materials section. Hydrolyze part of samples by mixing 480 µL of sample with 60 µL 1M NaOH and incubate at room temperature shaking at 500 rpm for 15 min. Neutralize with 60 µL 1M HCl.
6. Pre-incubation with mAb: Transfer 75 µL of each sample and standard dilution onto a fresh Nunc U-bottom microplate low binding. Prepare mAb depending on its specificity and concentration. Add 75 μL of mAb per well. Place on plate incubator at room temperature shaking at 500 rpm for 1 h.
7. Transfer of analyte and mAB: Wash the blocked Nunc MaxiSorp microtiter plate as in **step 3**. Transfer 100 μL mixture of preincubated analyte + mAB per well from U-bottom microplate to blocked MaxiSorp plate. Place on plate incubator at room temperature shaking at 500 rpm for 1 h.
8. Goat‐anti‐rat‐POD: Pipette 5 µL of GAR-POD to 40 mL of 40 mM PBST. Wash plate as in **step 3** and add 100 µL GAR-POD per well. Place on plate incubator at room temperature shaking at 500 rpm for 1 h.
9. Substrate: Prepare substrate by adding 600 µL TMB and 150 µL H2O2 to 37.5 mL substrate (acetate) buffer. Wash plate as in **step 3** and add 100 μL substrate per well for the HRP reaction. Incubation in the dark (e.g. drawer) for 5‐25 min. (*see* **Note 8**)
10. Stop reaction: Add 50 μL of 2M H2SO4 per well in the flue (*see* **Note 9**).
11. Absorbance measurement: Measure the optical density of the plate wells at 450nm (reference 650nm), auto mix before measurement with microplate reader.
12. Turn off washer: If you are using a washer, put washing buffer and all other buffers back in the refrigerator. Attach a bottle with ultrapure water to the washer tubing and wash until no foam is visible in the exhaustion tube. Empty the vacuum bottle and turn off the machine.
13. Evaluation: Curve fitting of the standard curve can be performed e.g. with SOFTmax Pro 5.2 (Molecular Devices), using the four parameter fit according to the equation [10]:

$$y=\frac{A-D}{1+ \left(\frac{x}{C}\right)^{B}}+D$$

where *x* is the sample concentration and *y* the measured optical density. *A* is the *y* value corresponding to the asymptote at low values of the x-axis and *D* is the *y* value corresponding to the asymptote at high values of the x-axis. The coefficient *C* is the *x* value corresponding to the midpoint between *A* and *D* (IC50). The coefficient *B* describes how rapidly the curve makes its transition from the asymptotes in the center of the curve.

[Here figure 1]

After *A*, *B*, *C*, and *D* have been determined experimentally, the equation can be used to calculate the sample concentration *x* from the measured optical densities:

$$x= \left(\frac{A-D}{y-D}-1\right)^{\frac{1}{B}} ×C$$

From these sum parameters the individual concentrations of HSL and HS can be calculated like this (for explanations and details see [8]):

$$c\left(HSL\right)= \frac{S(HSL)\_{hydrolyzed}-S(HSL)\_{non-hydrolyzed}}{CR\_{HS}-1}$$

$$c\left(HS\right)= S(HS)\_{non-hydrolyzed}-c\left(HSL\right)×CR\_{HSL}$$

*c(HSL)* and *c(HS)* are the individual concentrations of HSL and HS in the samples, while *S(HSL)* hydrolyzed and non-hydrolyzed and *S(HS)* non-hydrolyzed are the actually measured sample concentrations as sum parameters for the hydrolyzed and non-hydrolyzed samples. *CRHSL* and *CRHS* are the cross reactivity values calculated for each measured plate by dividing the IC50 values (equal to *C* in the equation above) of the standard curve for HS by the IC50 of the standard curve for HSL for *CRHSL* or vice versa for *CRHS* (*see* **note 10**).

### Acyl-HSL/HS extraction from cell pellets [[11](#_ENREF_11)]

1. Add 3 mL of acidified ethyl acetate to a cell pellet and stir for 10 minutes at room temperature.
2. Evaporate the solvent under a continuous nitrogen stream (*see* **Note 11**) at room temperature.
3. Reconstitute extract in 500 µL ethyl acetate-acetonitrile (50:50 %v/v) and keep it a 4oC for further analysis.

### Solid phase extraction (SPE) of supernatants [[7](#_ENREF_7)]

1. Weigh 25 mg of HMP in a glass vial for extraction of 2.5 mL cell culture supernatant or HSL/HS standards, respectively.
2. Capture HMP at the edge of the vial using an external magnet to discard wash solutions or culture medium after each step.
3. Condition HMP with 1 mL methanol and 1 mL purified water for 2 minutes, respectively.
4. Add 2.5 mL of cell culture supernatant or HSL/HS standard and incubate the mixture for 20 minutes at room temperature, stirring occasionally.
5. Wash HMP with 2 mL purified water.
6. Elute HSL with a 2 mL solvent mixture of 3-propanol and hexane (75:25 %v/v) and transfer the eluant into a 2 mL tube.
7. Dry the eluants using a centrifugal vacuum concentrator or evaporate solvent mixture under a low stream of nitrogen.
8. Re-dissolve extract in 250 µL acetonitrile-water (10:90 %v/v) and keep it cooled for further analysis.

### UHPLC-ESI-QToF-MS analysis

We used a Waters Acquity UPLC, consisting of binary solvent manager, tempered sample manager, tempered column manager and photo diode array detector, coupled to an electrospray ionization quadrupole time-of-flight mass spectrometer (ESI-QToF-MS, Bruker Daltonik).

1. Install reversed phase C18 column in the column manager and set the temperature to 40 °C.
2. Install eluants A and B on the binary solvent manager and purge all lines for 4 minutes with a flow rate of 8 mL/min. Equilibrate the C18 column starting with 100 % eluant B and a flow rate of 0.1 mL/min. Change solvent composition after 20 minutes first to 50 % eluant B and second to 0.5 % eluant B.
3. Set sample temperature to 4 °C to keep samples stable for the analysis.
4. Use the following settings for each chromatographic run: Apply a linear gradient from 0-90 % eluant B within 5 minutes and with a flow rate of 0.1 mL/min. Keep the solvent composition stable for 2.5 minutes, before equilibrating the column with 0.5 % eluant B. Inject 5.0 µL of sample. Perform UV detection at 195 nm with a scan rate of 10 Hz.
5. Acquire mass spectra in positive ionization mode within a mass range of 50-1000 m/z. (*see* **Note 12**). Measure HSL/HS standards from lower to higher concentrations first and then analyze samples in randomized order.
6. In order to ensure mass accuracy calibrate acquired spectra according to reference mass signals, e. g. from solvent impurities.
7. Create extracted ion chromatograms for protonated masses of HSL/HS molecules and integrate peaks to calculate peak areas (Fig. 2).

[Fig. 2 near here]

1. Plot peak areas of HSL/HS standards and make a linear equation. The coefficient of determination should be bigger than 0.950.
2. Calculate quantities of samples according to linear equation of HSL/HS standards.

## Notes

1. Put the solution for 5 minutes into an ultrasonic bath to ensure complete dissolution.
2. Oasis® HLB is a hydrophilic-lipophilic balanced reversed phase sorbent found and supplied by Waters GmbH, Milford, USA. It was tested to be the best sorbent for extracting acyl-HSL/HS from cell culture supernatants.
3. The reaction at 200 °C is forming high pressure according to boiling retardation of the solution. Therefore, usage of a tightly closed stainless steel digestion vessel is indispensable. Keep the vessel closed until it is cooled down to room temperature before continuing.
4. Use all solvents always at the same temperature (best room temperature) to prevent volumetric differences. To ensure correct volumetric percentages of organic solvents in mixtures, measure volumes by use of graduate flasks. Shake or stir organic solvent mixtures carefully to ensure complete mixing.
5. The concentration range depends on the limit of detection (LOD). It might be necessary to adjust it to the analytical instrument used for quantification. For correct quantification of HSL/HS from supernatant, standard solutions should be prepared following the same protocol for HMP-SPE. Standards for the quantification of HSL/HS from cell pellets can be prepared by dilution of a stock solution with acetonitrile. It is best to prepare fresh standard solutions each time. Mixing different HSL or HS standards in one solution is possible. HSL standards are commercially available as *N*-‘R’-L-homoserine lactones, *N*-3-oxo-‘R’-L-homoserine lactones and *N*-(3-hydroxy-‘R’)-L-homoserine lactone with ‘R’ as the aliphatic side chain length differing from C4 to C16.
6. Ultracentrifugation is only needed if HSLs/HSs should be quantified in intact cells. A detailed description is given by [[12](#_ENREF_12)]. For the quantification of excreted molecules in culture supernatants, a centrifugation at 15,000 g for 30 min is sufficient.
7. Samples should be stored no longer than a month depending on sample type and acyl-HSL concentration. For details see [[10](#_ENREF_10)].
8. Depending on the individual assay the incubation time varies. The darkest wells should be about the color of a transparent blue 1 mL pipette tip (equals approximately to an OD450 nm of 1.0). It is critical not to incubate for too long, as then the color signal will be in saturation and the standard curve will be flawed.
9. No washing step should be carried out before adding the stop solution. If you are handling more than one plate, make sure, that the incubation time - from adding the substrate until stopping the reaction - is the same for every individual plate. Usually it is sufficient to always keep the same order in handling the plates.
10. Make sure not to forget any dilution of the samples you might have applied. For example, the hydrolysis of the samples in 3.2. step 5 results in a dilution factor of 1.25. In highly concentrated samples, or samples with problematic matrix effect, dilution of the samples with PBS to up to 10% of the original concentration might overcome these difficulties.
11. You may also use a centrifugal vacuum concentrator at 30–35 °C.
12. Settings for MS detection: Dry gas flow 10 L/min, dry gas temperature 200 °C, nebulizer gas flow 2.0 bar, capillary voltage 4500 V, end plate offset -500 V, ion energy 3.0 eV, collision energy 8.0 eV. The MS was first calibrated on a reference standard including 5 masses within a mass range of 100-1600 m/z.

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## Caption Figure 1

Standard curves for the competitive ELISA detecting 3oxoC12-HSL and 3oxoC12-HS

## Caption Figure 2

Extracted ion chromatograms of *N*-butyryl-L-homoserine lactone, *N*-hexanoyl-L-homoserine lactone, *N*-octanoyl-L-homoserine lactone, *N*-decanoyl-L-homoserine lactone, *N*-dodecanoyl-L-homoserine lactone, *N*-tetradecanoyl-L-homoserine lactone (C4-C14; ***top***), *N*-(3-oxobutyryl)-L-homoserine lactone, *N*-(3-oxohexanoyl)-L-homoserine lactone, *N*-(3-oxooctanoyl)-L-homoserine lactone, *N*-(3-oxodecanoyl) -L-homoserine lactone , *N*-(3-oxododecanoyl) -L-homoserine lactone, *N*-(3-oxotetradecanoyl)-L-homoserine lactone (C4-C14; ***middle***) and *N*-(3-hydroxybutyryl)-L-homoserine lactone, *N*-(3-hydroxyhexanoyl)-L-homoserine lactone, *N*-(3-hydroxyoctanoyl)-L-homoserine lactone, *N*-(3-hydroxydodecanoyl)-L-homoserine lactone, *N*-(3-hydroxytetradecanoyl)-L-homoserine lactone (C4-C8, C12-C14; ***down***)