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Development and validation of a UHPLC-MS/MS procedure for quantification of the Pseudomonas Quinolone Signal in bacterial culture after acetylation for characterization of new quorum sensing inhibitors

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ABSTRACT

The appearance of antibiotic resistance requires novel therapeutic strategies. One approach is to selectively attenuate bacterial pathogenicity by interfering with bacterial cell-to-cell communication known as quorum sensing. The *pqs* quorum sensing system of *Pseudomonas aeruginosa* employs as signal molecule the Pseudomonas Quinolone Signal (PQS; 2-heptyl-3-hydroxy-4-(1*H*)-quinolone), a key contributor to virulence and biofilm formation. Thus, interference with PQS production is considered as promising approach for the development of novel anti-infectives. Therefore, in this study, we developed and validated an ultra-high performance liquid chromatographic-tandem mass spectrometric approach for reliable quantification of PQS in *P. aeruginosa* cultures for activity determination of new quorum sensing inhibitors. The poor chromatographic properties of PQS reported by others could be overcome by fast microwave-assisted acetylation. The validation procedure including matrix effects, recovery, process efficiency, selectivity, carry-over, accuracy and precision, stability of the processed sample, and limit of quantification demonstrated that the method fulfilled all requirements of common validation guidelines. Its applicability was successfully proven in routine testing. In addition, two-point calibration was shown to be applicable for fast and reliable PQS quantification saving time and resources. In summary, the described method provides a powerful tool for the discovery of new quorum sensing inhibitors as potential anti-infectives and illustrated the usefulness of chemical derivatization, acetylation, in liquid chromatography-mass spectrometry analysis.

Keywords: UHPLC-MS/MS; acetylation; Pseudomonas Quinolone Signal; quorum sensing inhibitors; validation; two-point calibration

1. Introduction

The emergence and spread of antimicrobial resistance in life-threatening pathogens such as *P. aeruginosa* are mainly attributed to the overuse of bactericidal antibiotics selecting for resistant mutants [1]. Moreover, bacteria living in biofilms exhibit higher resistance against antibiotics than planktonic cultures [2]. Thus, innovative therapeutic approaches such as anti-virulence and anti-biofilm strategies with the aim of reducing pathogenicity without affecting bacterial viability are in the focus of interest [3]. In this context, interference with bacterial quorum sensing (QS) has received special attention as potential therapeutic strategy for the development of novel anti-infective drugs [4,5]. QS functions *via* a cell-density dependent cell-to-cell communication system employing signal molecules that interact with response regulators thereby controlling the expression of genes *e.g.* involved in pathogenicity [6]. This allows the individual bacterial cells to coordinate group behaviors such as the production of virulence factors and biofilm formation.

The opportunistic human pathogen *P. aeruginosa* uses as signal molecules *N*-acyl-L-homoserine lactones (AHLs) for the *las*, *rhl* and 2-alkyl-4-(1*H*)-quinolones (AQs) for the *pqs* QS system [7]. The key player in the latter system is the Pseudomonas Quinolone Signal (PQS; 2-heptyl-3-hydroxy-4-(1*H*)-quinolone) [7], which is unique for *P. aeruginosa*. It acts as a strong agonist of the transcriptional regulator PqsR driving the expression of various virulence factors [8]. Furthermore, PQS down-regulates innate immune responses [9]. Regarding biofilms, PQS is supposed to function in the release of extracellular DNA, a main biofilm matrix component [10], thus promoting biofilm formation. The latter should be reduced by inhibiting PqsD, a key enzyme in PQS biosynthesis [11,12]. In fact, we could show recently that (2-nitrophenyl)(phenyl)methanol (2NPPM), a rationally designed inhibitor of PqsD, was able to reduce

PQS production and biofilm formation [13]. In addition, reduction of AQ production was accompanied by reduced bacterial dissemination and mortality in infected mice [14]. In summary, interception of QS by PQS interference is considered as a promising, novel approach for the development of anti-virulence and anti-biofilm compounds [15,16].

Therefore, a reliable analytical method is needed for the determination of PQS concentrations in bacterial cultures for discovery and biological evaluation of QS inhibitors (QSIs). To date, several approaches for quantification of PQS have been described, among them, methods not suitable for inhibitor testing such as unselective thin layer chromatography [17], capillary electrophoresis with cyclic voltammetry and amperometry [18], and time-consuming gas chromatography-mass spectrometry (GC-MS) [19]. Liquid chromatography-(tandem) mass spectrometry (LC-(MS/)MS) approaches for quantification of AQs have been described by the group of Rahme for various purposes using selected-ion monitoring [20,21], full-scan monitoring [22], or selected reaction monitoring (SRM) [14]. However, for their purposes, they used Erlenmeyer flasks (large scale) for cultivation, rather long run times with conventional LC separation (26 - 69 min) [20-22], and complex extraction procedures [22], which is too material- and time-consuming for routine inhibitor testing. Furthermore, simple addition of methanolic internal standard (IS) solution [20,21] may lead to contamination of column and ion source and increase the risk of ion suppression or enhancement by co-eluting substances. This would be disadvantageous for routine inhibitor testing.

Ortori *et al.* [23] reported a validated LC-MS/MS method for quantitative profiling of AHLs and AQs. However, PQS was described to show extremely poor peak shapes, independently of the used stationary phase [22,23], resulting in unquantifiable peaks and irreproducible results. To overcome this drawback, the chelator EDTA was added to the

mobile phase with the risk of increased ion suppression and contamination of the MS source.

During application of LC-MS/MS in routine testing [13,16], similar problems arose. Therefore, the development and validation of a fast ultra-high performance liquid chromatographic (UHPLC)-MS/MS procedure for reliable quantification of PQS in *P. aeruginosa* culture in the presence of QSIs will be presented with the aim of developing novel anti-infective drugs. Chemical derivatization, acetylation, was exploited to overcome the described chromatographic irreproducibility.

2. Materials and methods

2.1. Chemicals and bacterial strains

Acetic anhydride, ammonium acetate (both of analytical grade), and yeast extract were purchased from Fluka (Neu-Ulm, Germany), methanol, acetonitrile, ethyl acetate, sodium chloride, and trifluoroacetic acid (TFA) (all of analytical grade) from VWR (Darmstadt, Germany), pyridine (purissimum) and indole from Sigma Aldrich (Schnelldorf, Germany), peptone from casein from Merck (Darmstadt, Germany), and 2-amino-6-fluorobenzoic acid (6FABA) from Apollo Scientific (Stockport, UK). The following substances were synthesized as reported: PQS [16], PQS-d_4 [21], and 2NPPM [13] The *P. aeruginosa* strain PA14 and its isogenic *pqsH* transposon mutant were obtained from Prof. Susanne Häussler (Twincore, Hannover, Germany) and maintained in glycerol stocks stored at - 80°C.

2.2. Biosample for inhibitor assay

P. aeruginosa PA14 cultures were inoculated with a starting OD_{600} = 0.02 in 24-well Greiner Bio-One (Frickenhausen, Germany) Cellstar plates containing 1.5 mL of Luria-Bertani broth (0.5%, w/v, NaCl; 0.5%, w/v, yeast extract; 1%, w/v, peptone from casein) per well. Dimethylsulfoxide (DMSO) as a control or DMSO solutions of inhibitors were added to the cultures to a final DMSO concentration of 1% (v/v). Plates were incubated at 37°C, 200 rpm and a humidity of 75% for 16 h. For each inhibitor, cultivations were performed in triplicate [13]. To 500 µL of the cultures, 50 µL of a methanolic solution containing 100 µM of the IS PQS-d_4 were spiked in 2-mL Eppendorf tubes and processed as described below.

2.3. Biosample for method development and validation (blank sample)

P. aeruginosa PA14 isogenic *pqsH* mutant cultures were prepared as described for the inhibition assay. After incubation, the cultures from all wells were pooled, mixed and used freshly.

2.4. Sample processing

Ethyl acetate (1 mL) was added to the cultures samples, shaken on a rotary shaker for 5 min, and centrifuged for 5 min at 18,620 *g*. A 200-µL aliquot of the ethyl acetate phase was transferred to LC glass vials and evaporated to dryness at 35°C using a Thermo Fisher Scientific (TF, Dreieich, Germany) Savant SpeedVac.

The evaporated samples were acetylated in the glass vials with 200 µL of an acetic anhydride-pyridine mixture (3:2, v/v) for 10 min under microwave irradiation at 350 W according to the reported method [24]. After evaporation of the acetylation mixture at 460°C, the residues were dissolved in 400 µL of methanol.

2.5. Apparatus

The analyses were performed using a TF Accela UHPLC equipped with a degasser, a quaternary pump, and an autosampler (tray temperature 18°C) and coupled to a TF Accela photodiode-array detector (PDA) followed by a TF TSQ Quantum Access Max mass spectrometer with heated electrospray ionization source (HESI-II).

For gradient elution, a TF Accucore RP-MS column (150 x 2.1 mm, 2.6 µm) was used with a mobile phase consisting of ammonium acetate buffer (0.01 M) containing 1‰ TFA (v/v; eluent A; final pH 2.75) and acetonitrile containing 1‰ TFA (v/v; eluent B) and a flow rate of 800 µL/min under the following conditions: 0-6 min 5% to 100% A, 6-8 min hold, 8-8.1 min down to 5% A, 8.1-9 min hold for column equilibration with a total run time of 9 min. Column flushing was performed after 10 samples each with the same LC method. The injection volume was 5 µL using partial loop injection mode. The divert valve was set to 3 min.

The PDA was operated in the wavelength full scan mode for monitoring the inhibitors and PQS after acetylation at concentrations of 1 mM each.

The following MS conditions were used: electrospray ionization (ESI), positive mode, sheath gas, nitrogen at a flow rate of 50 arbitrary units; auxiliary gas, nitrogen at flow rate of 15 arbitrary units; collision gas, argon; vaporizer temperature, 550°C; ion transfer capillary temperature, 150°C; capillary offset, 35 V; spray voltage, 1500 V.

The mass spectrometer was operated in the SRM mode. Transitions and their particular settings, collision energy and tube lens, were determined using methanolic 10 μM solutions of PQS or PQS-d_4 injected after acetylation by the integrated syringe pump and obtained using the TSQ Tune Master software in the optimization MS+MS/MS mode.

According to the breakdown curves for diacetylated PQS (2acPQS; Fig. 1a), the transition of the precursor ion at *m/z* 344 to ion at *m/z* 260 was selected at collision energy of 27 V. Scan time was 0.1 s, scan width *m/z* 0.3, and tube lens offset 127 V. For 2acPQS-d_4 (Fig. 1b), the transition of the precursor ion at *m/z* 348 to ion at *m/z* 264 was selected at collision energy of 27 V. Scan time was 0.1 s, scan width *m/z* 0.1, and tube lens offset 133 V.

TF Xcalibur 2.2 software was used for data acquisition. The settings were as follows: peak detection algorithm, genesis; smoothing, 7; signal to noise threshold, 0.5. GraphPad Prism 5.00 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis.

The Fourier-transform infrared (FT-IR) spectrum of 2acPQS was recorded on a PerkinElmer (Rodgau, Germany) Spectrum 100 FT-IR spectrometer.

2.6. Preparation of stock solutions, calibration standards, and quality control samples

Methanolic stock solutions of PQS or PQS-d_4 were prepared at a concentration of 1 mM. For preparation of the calibration standards, spiking solutions were mixed containing 100 μM each of PQS-d_4 and 700, 525, 350, 175, 87.5, 21.88, 10.94, or 5.47 μM of PQS and for preparation of the quality control (QC) samples, spiking solutions containing 100 μM each of PQS-d_4 and 600, 450, 300, 150, 9.38, or 5.47 μM PQS. All

spiking solutions were made by adding the appropriate amount of the corresponding stock solution to obtain concentrations ten times higher than the final concentration in culture. Calibration standards and QC samples were prepared by adding 50 µL of the corresponding spiking solutions to 500 µL of blank samples and worked-up as described above.

2.7. Selectivity and carry over

The following samples were tested for peaks interfering with the detection of 2acPQS and/or 2acPQS-d_4 by monitoring the response in the 2acPQS-d_4 channel at regular concentration and/or in the 2acPQS channel at the lower limit of quantification (LLOQ): two processed blank samples each spiked with methanol, with PQS at the highest calibrator concentration (final concentration 70 µM), or with PQS-d_4 (zero sample) at regular concentration (final concentration 10 µM). In addition, with each batch of validation samples, two processed blank samples spiked with methanol and two zero samples each were analyzed in the same way.

Furthermore, carry over was investigated by analyzing a blank sample after injection of the processed highest calibrator by monitoring the responses as described before.

2.8. Matrix effect, recovery and process efficiency

Matrix effect, recovery, and process efficiency were determined according to the approach described by Matuszewski *et al*. [25]. Shortly, three sets of samples were prepared at 8 different concentrations (Table 1). Each sample was prepared in quadruplicate. Samples of set 1 represented the neat standard; samples of set 2 blank

samples spiked after extraction and samples of set 3 blank samples spiked before extraction. All samples were acetylated. Absolute peak areas of samples of set 2 were compared with those of set 1 to obtain matrix effect, of set 3 with those of set 2 to determine recovery, and of set 3 with those of set 1 to achieve process efficiency. In case of process efficiency and recovery, the values were corrected by the dilution factor.

2.9. Calibration model

Five replicates each of the calibration standards at each concentration level were analyzed as described above. The regression lines (linear through zero or ignoring zero) were calculated using a non-weighted, a weighted [1/concentration], and a weighted [1/(concentration)^2] least-squares regression model. Second-order models with the same weighting factors were also calculated. The following statistical tests using GraphPad software were performed: Grubbs' test for outliers, *F* test for heteroscedasticity, extra sum-of-squares *F* test for model comparison.

For each batch of validation samples, calibration curves were prepared using the same concentrations with single measurements per level. The back-calculated concentrations of all calibration samples were compared with their respective nominal values.

2.10. Full, one-point, and two-point calibration

Quantification was performed by comparing peak area ratios (2acPQS/2acPQS-d_4) to calibration curves generated by plotting the peak areas of spiked calibrators against their nominal concentrations. For full calibration, eight calibrators were used, for one-point

calibration, calibrators 1-8 each, and for two-point calibration, pairs of calibrators in all possible combinations.

2.11. Accuracy and precision

The QC samples were analyzed in sextuplicate at each concentration level on each of three days as recommended by the European Medicines Agency (EMA) guideline on bioanalytical method validation (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf) and processed as described above. The PQS concentrations in the QC samples were calculated *via* the corresponding calibration curves. Accuracy was expressed in terms of bias as the percental deviation of the mean calculated concentration at each concentration level from the respective nominal concentration, precision as the coefficient of variation (CV) as defined by EMA. Within-run accuracy and precision were calculated based on data obtained within one single run on each of the three days, between-run values based on mean data from the three days as defined by EMA.

2.12. Processed sample stability

The processed sample stability under the conditions of UHPLC-MS/MS analysis was estimated using QC samples at low (final concentration 0.938 µM) and high (final concentration 60 µM) concentrations. After extraction and derivatization, the samples were pooled at each concentration level and six aliquots each were injected under the conditions of a regular analytical run at time intervals of 4 h over a total run time of 16

1h. The QC samples were analyzed against a freshly prepared calibration curve and the calculated concentrations were compared to the nominal concentrations to obtain bias values as recommended by EMA.

2.13. Lower limit of quantification

The LLOQ was defined as the lowest point of the calibration curve, at which the response signal of 2acPQS was at least five times the signal of a blank sample and at which the acceptance criteria according to EMA for accuracy and precision were fulfilled.

2.14. Proof of applicability

The effect of 2NPPM (250 µM) [13] on the formation of PQS in *P. aeruginosa* PA14 cultures was tested three times independently using the described inhibition assay with two-point calibration. In addition, the inhibitor concentration to achieve a half-maximal degree of inhibition (IC_{50}) was determined twice independently for 6FABA [14] or indole [26] using at least five different inhibitor concentrations tested at least in duplicate. Dose-response curves were obtained by plotting the percental inhibition of PQS formation versus the logarithm of inhibitor concentration. IC_{50} values were calculated with GraphPad Prism 5.00 software using nonlinear regression (four-parameter dose-response model).

3. Results and discussion

3.1. Biosamples

For the biological evaluation of such inhibitors, a 24 well format-based bioassay using *P. aeruginosa* PA14 cultures was developed [13]. A PQS-deficient *P. aeruginosa* PA14 isogenic *pqsH* mutant culture [8] was chosen as biosample for method development and validation. As in *pqsH* mutants only the last step of PQS biosynthesis is interrupted, whereas the formation of the biosynthetic precursors is conserved [11,20,27], this culture was considered as the most appropriate biosample.

3.2. Sample processing

PQS was isolated from cultures by liquid-liquid extraction in order to reduce the risk of matrix effect and contamination of column and ion source. During method development, similar problems of unquantifiable peaks of PQS arose as described by Ortori *et al*. [23]. As the hydroxy function was discussed to be responsible for the poor chromatographic behavior of PQS [19,23], chemical derivatization, in common use for GC-MS analysis [24], was investigated. Microwave-assisted acetylation proved to be superior to other derivatization methods such as trimethylsilylation, methylation by diazomethane or methyl iodide (according to ref. [24], data not shown). The described acetylation method was optimized concerning type of reaction vessels, volumes, energy, and reaction time. In the context of matrix effect, recovery, and process efficiency testing (see below), the peak areas for set 1 showed with CVs < 11% over the whole calibration range good reproducibility of the derivatization step. This derivatization procedure was fast, reproducible, and allowed complete removal of the derivatization reagents avoiding contamination of the LC-MS system in contrast to EDTA addition used by Ortori *et al*.

1[23]. The resulting 2acPQS and 2acPQS-d_4 greatly improved the chromatographic 2properties (Fig. 2) and provided reproducible quantification results (see section 3.4.). 3The diacetyl products are depicted in Fig. 1. The 3,4-substitution pattern was supported 4by FT-IR spectroscopy, as a double band between 1770 and 1760 cm^{-1} corresponding to 5reported carbonyl stretching vibrations of similar quinolone-derived di-esters [28] was 6observed in the spectrum but no band at around 1650 cm^{-1} excluding the *N*-acetylated 7product [29] This study illustrates that chemical derivatization is a powerful tool also in 8LC-MS analysis as already applied *e.g.* in pharmacokinetics [30] to improve detection 9specificity, ionization efficiency, sensitivity and chromatographic behavior of analytes.

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11*3.3. Separation and detection by UHPLC-MS/MS*

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13A 6 min gradient was developed allowing fast sample analysis as well as separation of 14matrix components. Mass spectrometric detection was performed using positive ESI 15mode and monitoring the analytes in SRM mode. Ion at *m/z* 344 corresponded to the 16protonated parent mass of 2acPQS, whereas the respective mono- and non-acetylated 17fragments resulted in ions at *m/z* 302 and 260, respectively (Fig. 1a). Ions at *m/z* 188 and 18175 referred to fragments described for PQS by Lépine *et al*. [22]. For sensitivity 19reasons, transition *m/z* 344>260 was selected at collision energy of 27 V. The transition 20for the IS 2acPQS-d_4 was chosen accordingly (Fig. 1b). Only one transition was chosen 21in order to obtain enough data points for good reproducibility (even in two-point 22calibration).

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24*3.4. Method validation*

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This method was validated according to the EMA guideline on bioanalytical method validation wherever reasonable. In addition, the results for precision and accuracy obtained with full and one-point or two-point calibration were systematically compared.

3.4.1. Selectivity and carry over

In order to assess selectivity of the method, processed blank samples spiked with methanol, PQS, or PQS-d_4 were analyzed (Fig. 3). The areas of interfering peaks were lower than 20% of the peak areas of 2acPQS at LLOQ or 5% of the peak area of PQS-d_4 thus fulfilling the criteria of selectivity according to EMA. Only two processed blank samples each were tested because only one type of biomatrix was used, namely the bacterial culture grown under standardized conditions.

However, compounds to be tested for PQS inhibition should be checked for interference with 2acPQS and 2acPQS-d_4. In case of co-elution, possible ion suppression or enhancement should be excluded as described by Remane *et al*. [31].

Furthermore, carry over could be excluded as the response signals in the MS/MS channels of 2acPQS and 2acPQS-d_4 in the blank sample following the high concentration standard were within the acceptable ranges according to EMA. Therefore, no additional wash steps were required between two injections.

3.4.2. Matrix effect, recovery, and process efficiency

As given in Table 1, matrix effects were in the range of 94-113% with CVs < 10% indicating very low risk of interference even at low concentrations. In addition, the

values normalized by the IS PQS-d_4 were in the range of 99-102% with CVs < 2% and thus considered as negligible.

As further given in Table 1, the recoveries for PQS and PQS-d_4 ranged from 94-104% and 94-102%, respectively with CVs < 14%, and the process efficiencies from 92-112% and 92-110%, respectively with CVs < 12%. Thus, these results demonstrated good sample processing.

3.4.3. Calibration model

A calibration curve consisting of eight concentration levels with five replicates each was constructed to evaluate the calibration model. Considering an amount of about 60 µM PQS in incubated bacterial cultures (data not shown), a calibration range from 0.5-70 µM was chosen. Thus, reliable quantification of low PQS levels after strong inhibition should be possible.

Because of unequal variances (heteroscedasticity) across the calibration range, a 1/ [concentration]^2 weighted calibration model was chosen. Comparing different regression models, a second-order (quadratic) calibration turned out to be statistically the best. Nevertheless, two linear regression models (one forced through zero, one ignoring zero) were additionally used for assessing accuracy and precision in order to check whether one-point [32] or two-point [33] calibration could be applicable saving time and resources.

3.4.4. Accuracy and precision

For the calibration standards, the back-calculated concentrations fulfilled the acceptance criteria of EMA (accuracy: bias ≤ 15%, ≤ 20% at LLOQ; precision: CV ≤ 15%, ≤ 20% at LLOQ). For determination of accuracy and precision, the QC samples were analyzed in sextuplicate on each of 3 days. The obtained data applying full quadratic and the two full linear calibrations are given in Table 2. According to the EMA guidelines, the accuracy and precision data obtained after quadratic as well as after both full linear calibrations fulfilled the acceptance criteria (accuracy: bias ≤ 15%, ≤ 20% at LLOQ; precision: CV ≤ 15%, ≤ 20% at LLOQ).

For one-point calibration, accuracy and precision data for the QC samples were calculated using calibrators 1-8 each as one-point calibrator. Calibrator 4 showed best accuracy and precision data, which were therefore statistically compared to those obtained by full calibration forced through zero as described by Remane *et al*. [34]. Briefly, the corresponding bias values (neglecting signs) were subjected to *t* tests and the correspondent variance values submitted to *F* tests. Both tests indicated that full calibration was significantly superior to one-point calibration. Thus, one-point calibration was not considered to be acceptable [32].

In order to evaluate two-point calibration, accuracy and precision data for the QC samples (Table 3) were calculated using calibrator pair 1/6, chosen according to Tan *et al*. [33]. These data were systematically compared to the respective results calculated with full linear calibration ignoring zero as described above, as this calibration was superior to that through zero. Neither the *t* tests nor the *F* tests provided significant differences ($p<0.05$) at all concentration levels. Thus, two-point calibration ($y = 230.0001042x + 0.009397$) was accepted as the method of choice for routine testing saving time and resources [33].

3.4.5. Stability of the processed sample

In the processed QC samples, 2acPQS and 2acPQS-d_4 were stable for a period of at least 16 h at autosampler conditions of +18°C as the bias values were less than 6% thus fulfilling the EMA acceptance criteria. Long term and freeze/thaw stability tests were not performed because for routine inhibitor testing samples are always freshly prepared and do not need to be stored for a long time or subjected to freeze-thaw cycles.

3.4.6. Lower limit of quantification

For the lowest calibrator, the analyte response was more than 5 times the response of a blank sample and accuracy and precision data fulfilled the EMA acceptance requirements (accuracy: bias ≤ 15%, ≤ 20% at LLOQ; precision: CV ≤ 15%, ≤ 20% at LLOQ). Thus, the method allowed the detection of low PQS levels after strong inhibition of its formation.

3.5. Application

The new approach was tested for applicability by analyzing the effect of 2NPPM, a known QSI, on extracellular PQS levels in *P. aeruginosa* PA14 liquid cultures [13]. In Fig. 2, chromatograms are depicted of SRM transition *m/z* 344>260 indicating 2acPQS and transition *m/z* 348>264 indicating 2acPQS-d_4 in a processed *P. aeruginosa* PA14 culture biosample after incubation without (a) or with 2NPPM (b). In accordance to ref. Storz [13], PQS formation was inhibited by 37±6% at an inhibitor concentration of 250 µM. In addition, dose-dependent effects of the known QSIs 6FABA [14] and indole [26]

on PQS production were tested providing the following IC_{50} values: 109 (± 14) µM for 6FABA, and 236 (± 6) µM for indole (Fig. 4). 6FABA nearly abolished PQS production at a concentration of 1.5 mM in accordance with the literature [14]. Tashiro *et al.* [26] reported a concentration of indole for half-maximal inhibition of PQS synthesis of around 50 µM, which was lower than the IC_{50} value obtained in this study. However, they used a different *P. aeruginosa* strain (PAO1) that produced less PQS than PA14 [21]. Separation of the acetylated inhibitors from 2acPQS and 2acPQS-d_4 was demonstrated using the same LC system with PDA detection because the acetylated inhibitors were not sufficiently ionized under the applied LC-MS conditions.

4. Conclusions

The described UHPLC-MS/MS method enables selective detection and accurate and precise quantification of PQS in bacterial culture for monitoring the anti-QS activity of test compounds. The procedure copes with the criteria of a validated method and was shown to be efficient in routine application. The use of a two-point calibration in combination with a simple and fast sample work-up allowed time- and cost-efficient assay performance. Improvement of LC properties was a further example for the advantageous use of chemical derivatization in LC-MS analysis. In summary, the described approach is a useful tool in the discovery of new QSIs as potential anti-infectives.

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Figure captions

Fig. 1. Structures and breakdown curves of the precursor ion at *m/z* 344 for 2acPQS (a) and of that at *m/z* 348 for 2acPQS-d_4 (b).

Fig. 2. Chromatograms of SRM transition *m/z* 344>260 indicating 2acPQS and transition *m/z* 348>264 indicating 2acPQS-d_4 in a processed *P. aeruginosa* PA14 culture biosample after incubation without (a) or with 2NPPM (b).

Fig. 3. Chromatograms of SRM transition *m/z* 344>260 (2acPQS) and transition *m/z* 348>264 (2acPQS-d_4) of processed blank samples spiked with methanol (a), with 70 µM PQS (b), or with 10 µM PQS- d_4 (zero sample).

Fig. 4. Dose-response plots for the QSIs 6FABA (a) and indole (b). Mean values of n ≥ 2, error bars represent standard deviation.

Table 1 Calibrator number, nominal concentration, matrix effect, recovery, and process efficiency data (mean of n = 4, CV %)

Calibrator number	Nominal concentration [nM]	Matrix effect % (CV %)			Recovery % (CV %)		Process efficiency % (CV %)	
		PQS	PQS-d_4	PQS/PQS-d_4	PQS	PQS-d_4	PQS	PQS-d_4
1	**547**	113 (8)	111 (8)	102 (0)	100 (4)	96 (4)	112 (10)	106 (10)
2	**1094**	112 (10)	112 (10)	101 (2)	94 (2)	92 (2)	110 (12)	106 (12)
3	**2188**	109 (1)	109 (2)	100 (1)	96 (3)	94 (3)	104 (4)	102 (5)
4	**8750**	105 (8)	106 (8)	99 (1)	98 (7)	96 (7)	102 (3)	100 (3)
5	**17500**	103 (3)	104 (3)	99 (1)	98 (4)	98 (4)	102 (4)	102 (4)
6	**35000**	97 (7)	98 (8)	99 (1)	96 (13)	94 (14)	92 (8)	92 (7)
7	**52500**	107 (6)	108 (6)	99 (1)	104 (2)	102 (2)	110 (8)	110 (7)
8	**70000**	94 (3)	95 (3)	99 (1)	102 (4)	100 (5)	96 (7)	94 (8)

Table 2 Nominal concentration, within- and between-run accuracy, within- and between-run precision for QC samples 1-6 (n = 6) calculated using full $1/x^2$ weighted quadratic, linear (forced through zero), or linear (ignoring zero) calibration

Calibration model			Full calibration, quadratic, $1/x^2$						Full calibration, linear, force, $1/x^2$						Full calibration, linear, ignore, $1/x^2$					
QC sample			**QC 1**	**QC 2**	**QC 3**	**QC 4**	**QC 5**	**QC 6**	**QC 1**	**QC 2**	**QC 3**	**QC 4**	**QC 5**	**QC 6**	**QC 1**	**QC 2**	**QC 3**	**QC 4**	**QC 5**	**QC 6**
Nominal conc. [µM]			0.547	0.938	15	30	45	60	0.547	0.938	15	30	45	60	0.547	0.938	15	30	45	60
Accuracy Bias [%]	**Within-run**	**Day 1**	-11.8	-8.9	-1.5	-1.0	-0.2	0.4	6.6	0.3	-5.3	-6.0	-6.2	-6.5	-12.7	-8.7	-0.5	-0.8	-0.9	-1.1
		Day 2	-6.6	-3.5	3.4	3.9	4.4	4.9	11.1	5.1	-0.7	-1.3	-1.6	-1.9	-7.2	-3.3	4.2	4.0	3.8	3.6
		Day 3	3.3	1.5	2.2	2.6	3.4	3.9	15.7	7.7	-0.5	-1.1	-1.2	-1.5	2.8	1.8	3.0	2.7	2.7	2.5
	Between-run		-5.0	-3.6	1.4	1.8	2.5	3.1	11.1	4.3	-2.2	-2.8	-3.0	-3.3	-5.7	-3.4	2.2	2.0	1.9	1.7
Precision CV [%]	**Within-run**	**Day 1**	0.9	0.6	0.6	0.7	0.7	0.6	0.7	0.5	0.6	0.7	0.7	0.6	1.0	0.6	0.6	0.7	0.7	0.6
		Day 2	1.5	1.0	0.7	0.5	0.7	0.5	1.2	0.9	0.7	0.5	0.7	0.5	1.5	1.0	0.7	0.5	0.7	0.5
		Day 3	1.6	1.4	1.0	1.0	1.0	1.3	1.4	1.3	1.0	1.0	0.9	1.3	1.7	1.4	1.0	1.0	0.9	1.3
	Between-run		8.0	5.4	2.5	2.5	2.4	2.3	4.1	3.6	2.8	2.9	2.9	2.9	8.3	5.5	2.4	2.4	2.4	2.4

Table 3 Nominal concentration, within- and between-run accuracy, within- and between-run precision for QC samples 1-6 (n = 6) calculated using one-point or two-point calibration

Calibration model			One-point calibration (calibrator 4)						Two-point calibration (calibrator pair 1/6)					
QC sample			QC 1	QC 2	QC 3	QC 4	QC 5	QC 6	QC 1	QC 2	QC 3	QC 4	QC 5	QC 6
Nominal conc. [µM]			0.547	0.938	15	30	45	60	0.547	0.938	15	30	45	60
Accuracy Bias [%]	Within-run	Day 1	9.5	3.0	-2.7	-3.4	-3.7	-4.0	-12.3	-8.3	-0.1	-0.4	-0.5	-0.7
		Day 2	14.4	8.1	2.2	1.5	1.1	1.0	-7.0	-3.2	4.2	4.0	3.6	3.6
		Day 3	17.6	9.5	1.2	0.6	0.2	0.2	2.7	1.9	-3.1	-2.8	-2.6	-2.6
	Between-run		13.8	6.9	0.2	-0.4	-0.5	-0.9	-5.5	-3.2	-2.4	-2.1	-2.1	-1.8
Precision CV [%]	Within-run	Day 1	0.7	0.5	0.6	0.7	0.7	0.6	1.0	1.0	0.6	0.7	0.7	0.6
		Day 2	1.2	0.9	0.7	0.5	0.7	0.5	1.5	1.0	0.7	0.5	0.7	0.5
		Day 3	1.4	1.3	1.0	1.0	0.6	1.3	1.7	1.4	1.0	1.0	0.6	1.3
	Between-run		3.6	3.2	2.6	2.6	2.7	2.7	8.1	5.3	2.1	2.2	2.2	2.2