Identification and Quantification of Secondary Metabolites by LC-MS from Plant-associated *Pseudomonas aurantiaca* and *Pseudomonas chlororaphis*

Izzah Shahid[1], Muhammad Rizwan[2] and Samina Mehnaz[1, *]

[1]Department of Biological Sciences, Forman Christian College (A Chartered University), Lahore, Pakistan; [2]Department of Chemistry, Government College Township, Lahore, Pakistan

*For correspondence: saminamehnaz@fccollege.edu.pk

[Abstract] Increased antibiotic resistance of plants and human pathogens and continuous use of chemical fertilizers has pushed microbiologists to explore new microbial sources as potential antagonists. In this study, eight strains of *Pseudomonas aurantiaca* and *Pseudomonas chlororaphis*, have been isolated from different plant sources and screened for their antagonistic and plant growth promoting potential (Shahid *et al.*, 2017). All strains were compared with reference strain PB-St2 and their secondary metabolites were isolated by the use of solvent partitioning and subjected to LC/ESI/MS for confirmation of compounds. The ESI-mass spectra obtained were used to characterize the surfactants ionization behavior and $[M + H]^+$ and $[M + Na]^+$ ions were monitored for phenazines, derivatives of lahorenoic acid and cyclic lipopeptide (WLIP). LC-MS and HPLC methods were developed to see the elution of dominant metabolites in a single run to avoid the labor and separate methods of detection for all compounds. The method was found suitable and distinctively separated the compounds at different retention times in gradient flow. This method can be helpful to explore the metabolome of *Pseudomonas* sp. overall and in identification and quantification of strain specific metabolites.

Keywords: Phenazines, White-line inducing principle (WLIP), Lahorenoic acid, *Pseudomonas aurantiaca*, *Pseudomonas chlororaphis*, LC-MS

[Background] Authors in this research group are working on bioformulations and have isolated a large number of bacteria, including pseudomonads with potential plant growth promoting abilities. Pseudomonads are known for their biocontrol ability through antibacterial and antifungal secondary metabolites. These secondary metabolites successfully counter several phytopathogens of economically important crops and *Pseudomonas* based biofertilizers developed so far, have been marketed as commercial products (Mandryk *et al.*, 2007; Rovera *et al.*, 2014). *Pseudomonas aurantiaca* and *P. chlororaphis* strains are well known biocontrol agents due to the production of phenazines and cyclic lipopeptides and their broad spectrum antagonistic activities. Although many compounds have been reported by *Pseudomonas aurantiaca and P. chlororaphis* to date, the list of unidentified compounds is long (Chin *et al.*, 2001; Hu *et al.*, 2014).

Major metabolites produced by our strains were, white line inducing principle (WLIP), 2-hydroxy phenazine (2-OH-PHZ), phenazine-1 carboxylic acid (PCA) and Lahorenoic acid A. Previously reported methods for the detection of these compounds usually account only for HPLC, and comparison of

results with standards. Moreover, detailed information about their MS/MS spectra and LC-MS methods is not available. Many researchers described the short HPLC runs for the detection of phenazines that did not account for the metabolites that elute at later stages (El-sayed *et al.*, 2008; Upadhyay and Srivastava, 2011).

Lahorenoic acid A was exclusively reported by our group (Mehnaz *et al.*, 2013) and since then has not been reported by any other group. WLIP was also reported first time from *Pseudomonas aurantiaca* PB-St2 in the same paper, though it has been reported earlier from other species of *Pseudomonas* (Meng *et al.,* 2014). Keeping these findings in mind, a method was established that accounts for all the basic details in detection of these compounds from *Pseudomonas aurantiaca* in particular and other pseudomonads in general. LC-MS method was established with long run time and gradient flow, to make the better separation of these compounds. For confirmation of results, MS/MS fragmentation patterns of all compounds were also observed. The same LC-MS method was used for HPLC detection of these compounds, however, a different column was used. Many research groups working on metabolomics do not have access to standards of every compound. It was difficult to get the standards by our group as well and therefore, purified fractions of these compounds manually collected through HPLC, were used as reference standards for relative quantification of detected compounds. This method is significant as it provides the details about four essential and main secondary metabolites of *Pseudomonas* sp. in a single LC-MS and HPLC run.

Materials and Reagents

1. Pipette tips (Axygen™ 1,000 µl Universal Pipette Tips) (Corning, Axygen®, catalog number: T-1000-B)
2. Sterile syringe filters of 0.2 µm (Corning, catalog number: 431225)
3. Pasteur pipettes (*e.g.*, Fisherbrand™ disposable borosilicate glass Pasteur pipettes, Fisher Scientific, catalog number: 13-678-20A)
4. pH indicator strips (pH 0-14 Universal Indicator) (Merck, catalog number: 1095350001)
5. Nine bacterial strains including eight isolates of *Pseudomonas chlororaphis* subsp. *aurantiaca* (GS-1, GS-3, GS-4, GS-6, GS-7, ARS-38, PBSt-2, FS-2) and one *Pseudomonas chlororaphis* subsp. *chlororaphis* (RP-4) isolate
6. Hydrochloric acid (HCl), 36.5-38% (Sigma-Aldrich, catalog number: 320331)
7. Ethyl acetate, EMSURE® ACS, ISO, Reag. Ph Eur. (Merck, catalog number: 1096232500)
8. Sodium sulfate, anhydrous, ≥ 99% (Sigma-Aldrich, catalog number: 1614807)
9. Methanol gradient grade for liquid chromatography, LiChrosolv® Reag. Ph Eur (Merck, catalog number: 1060072500)
10. Chloroform, EMSURE® ACS, ISO, Reag. Ph Eur. (Merck, catalog number: 1024452500)
11. Water, LC-MS grade, LiChrosolv® (Merck, catalog number: 1153332500)
12. Dimethyl sulfoxide (DMSO), ≥ 99% (Sigma-Aldrich, catalog number: 472301)

13. Formic acid, 98-100%, EMSURE® ACS, ISO, Reag. Ph Eur. (Merck, catalog number: 1002640510)

14. Acetonitrile gradient grade for liquid chromatography, LiChrosolv® Reag. Ph Eur (Merck, catalog number: 1000302500)

15. Protease-peptone, microbiology grade (Sigma-Aldrich, catalog number: 82450)

16. Glycerol, molecular biology grade, ≥ 99.5% (Sigma-Aldrich, catalog number: G9012)

17. Potassium phosphate dibasic (K_2HPO_4), ACS reagent, ≥ 98% (Sigma-Aldrich, catalog number: P3786)

18. Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), ACS reagent, ≥ 98% (Sigma-Aldrich, catalog number: 230391)

19. Agar (for solid medium only), powder for Microbiology (Sigma-Aldrich, catalog number: 05040)

Equipment

1. Microbiological static incubator (*e.g.*, Memmert, model: IF55 standard incubator)

2. Microbiological incubator shaker (*e.g.*, IKA, model: KS 4000 i control)

3. Pipettes (*e.g.*, Sartorius, catalog numbers: 728020, 728050, 728060 and 728070)

4. Large-volume centrifuge (*e.g.*, HERMLE Labortechnik, model: Centrifuge ZK 496)

5. Erlenmeyer flasks (*e.g.*, PYREX™, 1,000 ml, 500 ml, Corning, PYREX®, catalog number: 4980-1L)

6. Separatory funnels with stopcock and ring stand (*e.g.*, 1,000 ml, 500 ml, VWR, catalog number: 89426-640)

7. Autoclave

8. Glass beakers (*e.g.*, PYREX™, 1,000 ml, 500 ml, 100 ml, 50 ml, DWK Life Sciences, KIMBLE, catalog number: 14000-2000)

9. Glass stirrer

10. Rotary evaporator (*e.g.*, Buchi Rotavapor® R-100 (BÜCHI Labortechnik, model: Rotavapor® R-100) with Re-circulating Chiller F-100/F-105 (BÜCHI Labortechnik, model: Recirculating Chiller F-100/F-105), Vacuum Pump V-100 (BÜCHI Labortechnik, model: Vacuum Pump V-100), water bath and Interface I-100 (BÜCHI Labortechnik, model: Interface I-100))

11. Fume hood (*e.g.,* Labconco, model: 4' Protector XStream Laboratory Hood, catalog number: 110410000)

12. LC-MS (*e.g.*, Thermo Finnegan LC-MS, ESI Ion Trap Mass Spectrometer, Thermo Electron, model: LCQ Advantage Max)

13. TLC plates (Silica Gel 60G F_{254} 20 x 20 cm, *e.g.,* Merck, catalog number: 100390)

14. C18 column for MS (*e.g.*, Thermo Hypersil Gold C18 column, Length 250 mm, 4.6 mm ID, 5 μm particle size, Thermo Fisher Scientific, catalog number: 25005-254630, Lot No. 7648, S/N 0871381M)

15. HPLC system (*e.g.*, WATERS, model: e2995 Separations Module, 2998 Photodiode Array (PDA) Detector)

16. HPLC column (*e.g.*, Nucleosil C18 column, 4.6 x 250 mm, 5 µM; Macherey-Nagel, Germany)

Software

1. Xcalibur 2.0 software (Xcalibur™ Software, control and process data from LC-MS instruments. It is Windows™ based software that provides method setup, data acquisition, data processing and reporting. Data files are retrieved in Qual Browser and are processed for interpretation and analysis)

Procedure

A. Culture conditions

1. Total nine bacterial strains including eight isolates of *Pseudomonas chlororaphis* subsp. *aurantiaca* (GS-1, GS-3, GS-4, GS6, GS-7, ARS-38, PBSt-2, FS-2) and one isolate *Pseudomonas chlororaphis* subsp. *chlororaphis* (RP-4) were included in this research. These bacterial strains are 16S rRNA identified and screened for their PGPR activities (Shahid *et al.*, 2017). All bacterial strains were streaked on King's B agar plates (see Recipes) and plates were incubated at 28 ± 2 °C for 48 h.

2. A single colony of each bacterial strain; GS-1, GS-3, GS-4, GS6, GS-7, ARS-38, PBSt-2, FS-2 and, RP-4 was separately used to inoculate 10 ml of King's B broth. Cultures were incubated in a shaking incubator for 24 h at 150 rpm and 28 ± 2 °C. Next day, each bacterial culture was individually inoculated (2%) in 500 ml King's B broth and flasks were incubated at 28 ± 2 °C, 150 rpm for 96 h.

B. Extraction procedure

1. After 96 h of incubation, harvest cultures and centrifuge at 3,376 *x g* for 40 min at 4 °C. Collect supernatants in separate flasks.

2. All supernatants are acidified to pH 2 with 1 N HCl and pH is monitored with pH-indicator strips.

3. Acidified supernatants are extracted twice with equal volume of ethyl acetate. For this, in each flask with 500 ml of acidified supernatant, add 500 ml of ethyl acetate. Put flasks in a shaking incubator for 10-15 min and then transfer materials into separatory funnels. The materials will separate into two phases, organic phase and water phase. The upper phase is organic phase while the lower phase has culture supernatant. Collect them into separate beakers. Re-extract the collected culture supernatant with ethyl acetate and combine the re-extracted organic layer into the previous collected organic phase.

4. Add 20-30 g of anhydrous sodium sulphate to the beaker with organic phase and stir with a glass stirrer. This step is essential to remove any moisture from the organic layers. Then, transfer this content to another clean and dry beaker (avoiding any salt particles) and finally in the rotary evaporator flask.

5. Turn on all basic units of rotary evaporator including central interface, glass assemblies, water bath, chiller, and vacuum. Set water bath temperature to 40-45 °C, and adjust rotary rotations accordingly to prevent any bumping of liquid in glass assemblies. When the liquid phase is completely dried, separate the round bottom flask with dried residue from rotary evaporator glass assembly.

6. Re-dissolve residues of extracts in methanol and chloroform (2 ml methanol:2 ml chloroform), completely dry in a fume hood and store at 4 °C.

C. Identification of bacterial compounds using LC-MS

1. For characterization of bacterial secondary metabolites, dissolve the extracts in 1.5 ml of methanol and 500 µl of chloroform.
   Note: Please be sure that extracts are completely dissolved and no un-dissolved residue left in the vials. If any portion of extracts remains undissolved, collect the methanol and chloroform soluble part in separate vials and dissolve the remaining undissolved part in DMSO (Dimethyl sulfoxide).

2. Take 500 µl of these extracts and individually filter with sterile syringe filters of 0.2 µm (Fisher Scientific).

3. Subject the extracts of all strains to Liquid Chromatography-Electrospray Ionization-Mass Spectrometry (LC/ESI/MS), for identification of secondary metabolites.

4. Set up the instrument and perform LC-ESI MS/MS runs using a Thermo Finnegan HPLC system, coupled to an LCQ Advantage Max ESI-Ion Trap Mass Spectrometer (Thermo Electron, USA).

5. Chromatographic separations are achieved using Thermo Hypersil Gold C18 column (4.6 x 250 mm, 5 µm particle size). Set the temperature of the column compartment at 25 °C, and load 20 µl of injection volume on the column.

6. A gradient used to separate the metabolites consists of two solvent systems. Solvent A is 0.1% formic acid in water and solvent B is 0.1% formic acid in acetonitrile. Total LC-MS/MS runtime is 55 min, and set the flow rate at 0.7 ml/min.

D. Gradient conditions of LC-MS/MS

1. Set gradient conditions as follows (Table 1):

Table 1. Gradient run used for LC-MS and HPLC analyses

Time	H₂O %	Acetonitrile %
0.00	95	5
1.00	95	5
37.0	5	95
42.00	5	95
45.00	95	5
55.00	95	5

2. ESI positive mode is used for the runs with data dependent protocol with a mass range of 150-1,500 a.m.u. and two scan events are employed for this data. The first scan event is a full scan of 150-1,500 and the second scan is dependent on the most abundant ions in the first scan triggering their MS^2 acquisition.

3. Data are acquired at the normalized collision energy of 30%. The heated capillary is maintained at 350 °C, and sheath and auxiliary/sweep gases are at 60 and 25 arbitrary units, respectively.

4. Set the source voltage to 4.5 kV with 10 V capillary voltage. The ESI-mass spectra obtained are used to characterize the surfactant ionization behavior; [M + H]$^+$ and [M + Na]$^+$ ions are monitored for phenazines, *i.e.*, 2-hydroxy-phenazine(2-Oh-Phz) and phenazine-1-carboxylic acid (PCA); Lahorenoic acid A, and cyclic lipopeptide (WLIP). In addition, the ESI-MS/MS fragmentation behavior of identified peaks is investigated to confirm the structure of these secondary metabolites. Figures (Figures 1-3 and Supplemental Figures S1-S10) describe the structures, extracted ion current (XIC) chromatograms, mass spectrums and MS/MS fragmentation behavior of detected metabolites. Chemical formulas, exact masses and observed m/z values for detected metabolites have been given in Table 2.
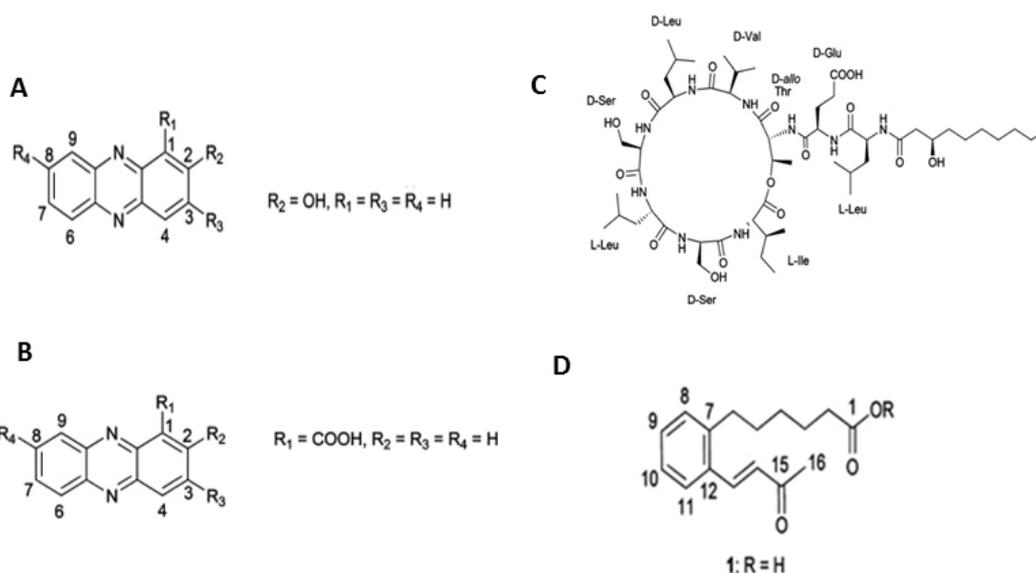
Figure 1. Structures of the compounds isolated from *Pseudomonas aurantiaca* and *Pseudomonas chlororaphis*. A. 2-hydroxyphenazine, B. phenazine-1-carboxylic acid, C. white-line-inducing principle, D. lahorenoic acid A (Mehanz *et al.,* 2013).

Table 2. Chemical formulas, exact masses and observed *m/z* values for detected metabolites

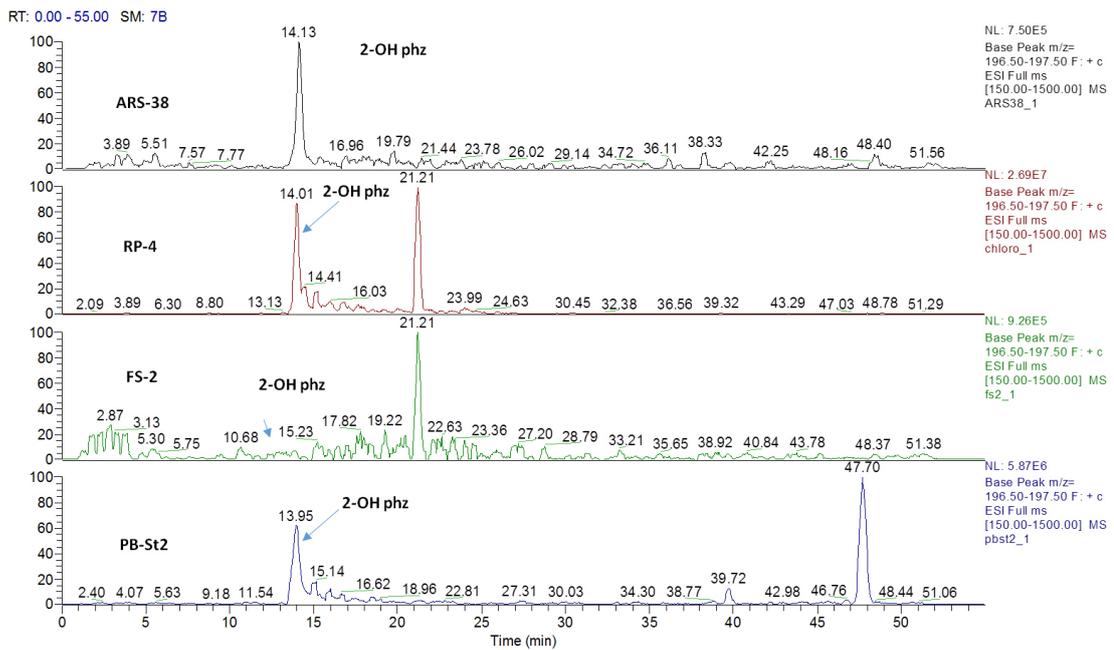| Metabolites	Chemical formula	Exact mass	Observed peaks *m/z*	
			[M + H]+	[M + Na]+
2-Hydroxyphenazine (2-OH-Phz)	$C_{12}H_8N_2O$	196.06	197.07	219.04
Phenazine-1-carboxylic acid (PCA)	$C_{13}H_8N_2O_2$	224.05	225.06	247.03
White-line-inducing principle (WLIP)	$C_{54}H_{95}N_9O_{16}$	1125.68	1126.69	1148.66
Lahorenoic acid A	$C_{16}H_{20}O_3$	260.14	261.14	283.12

Figure 2. Extracted ion current (XIC) chromatograms for 2-hydroxyphenazine (2-OH-Phz), *m/z* 197, [M + H]+ of four strains; ARS-38, RP-4, FS-2 and PB-St2

Figure 3. Mass spectrum of 2-Hydroxyphenazine, *m/z* [M + H] + 197.07

E. Thin layer chromatography (TLC) of identified secondary metabolites

Identified four secondary metabolites can also be analyzed by thin layer chromatography (TLC), using PB-St2 as a reference strain. PB-St2 is used as reference strain as all of its secondary metabolites have previously been published (Mehnaz *et al.*, 2009 and 2013).

1. For thin layer chromatography, load methanol fractions of all bacterial extracts (10 µl) on TLC plates (Silica Gel 60G F_{254} 20 x 20 cm). Mobile phase contains chloroform: acetone: acetic acid (78.4:20:1.6, v/v/v) and samples are spot inoculated on TLC plate for separation (Figure 4).

2. Air dry TLC plates (approximately for 5-10 min) and analyze for showing the separation of phenazines, *i.e.*, phenazine-1-carboxylic acid (PCA) and 2-hydroxy phenazine (2-OH-Phz).
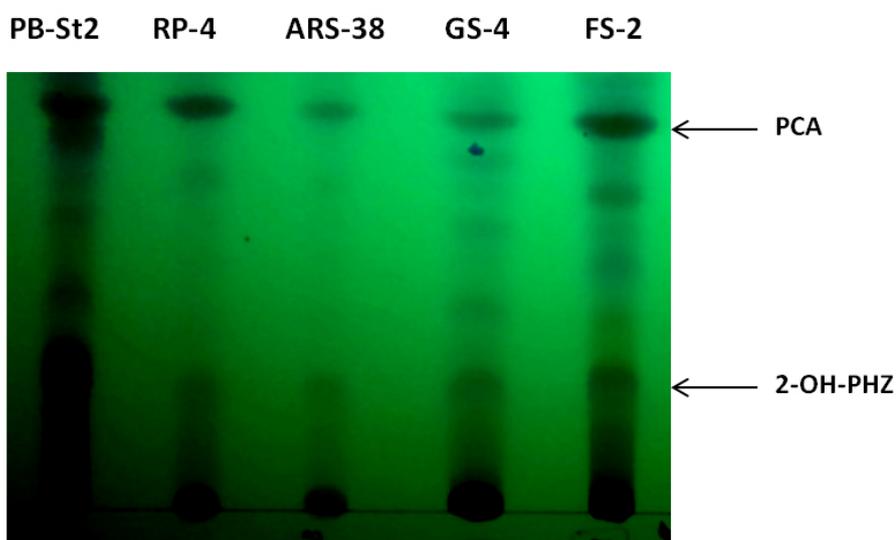

Figure 4. Thin layer chromatogram of phenazine-1-carboxylic acid (PCA) and 2-hydroxy phenazine (2-OH-PHZ), present in cell-free supernatants of bacterial extracts of RP-4, ARS-38, GS-4 and FS-2. PB-St2 fractions were used as reference standard for this TLC analysis.

F. Quantification of secondary metabolites

1. Manually collect the pure fractions of these compounds using HPLC from PB-St2 *Pseudomonas aurantiaca* isolate (or any bacterium that needs to be analyzed for its secondary metabolites) and analyze on LC-MS for their purity (Figure 5). The method for LC-MS is the same as used in HPLC analysis. The total run time is 55 min, with 20 µl injection volume and with same acetonitrile and water gradient run, as described in Table 1.

2. Analyze samples on Waters HPLC System (e2995, separations module) with 299 h photodiode-array (PDA) detector using a Nucleosil C18 column (4.6 x 250 mm, 5 µM; Macherey-Nagel, Germany). Collected fractions of these compounds are used as reference standard to quantify the production of these compounds from all bacteria used in this study.
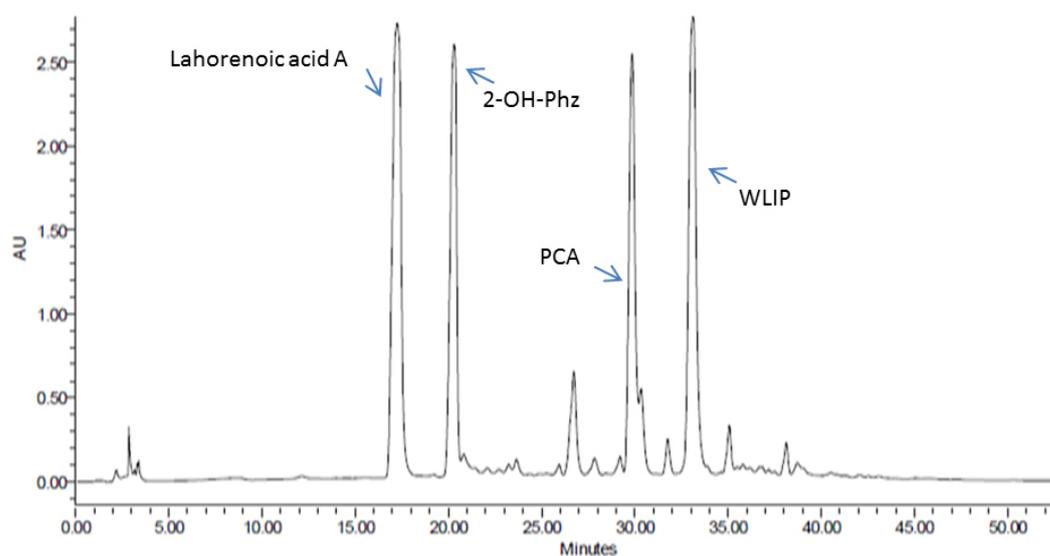
Figure 5. HPLC analysis of enriched *P. aurantiaca* PB-St2 fractions of detected metabolites

Data analysis

All LC-MS/MS data were acquired and data files were processed using Xcalibur 2.0 software. Data files were initially open using Xcalibur FTMS raw data files. These files displayed total ion chromatogram (TIC) on the top and m/z spectra of total scan at the bottom. Using software's basic features, data were acquired for reported four compounds and their ESI-MS/MS fragmentation patterns were also observed to confirm the structure of these secondary metabolites. ESI-MS/MS fragmentation was also confirmed with Wishart Research Group's (CFM-ID), Competitive Fragmentation Modeling for Metabolite Identification (cfmid.wishartlab.com).

Notes

1. Avoid any plastic equipment during the preparation of samples for HPLC or LC-MS and also at the time of extraction. This may add some plasticizers in your prepared samples that often result in false peaks in LC-MS and HPLC run.
2. Amount of the culture medium can also be reduced accordingly. This method used the fractions extracted from 500 ml of King's B broth. It can be reduced to 100 ml or 50 ml according to the requirement. Amount of organic solvents used will also get reduced with it.
3. For extraction with ethyl acetate, the supernatant is acidified to pH 2, while using dichloromethane (DCM) for extraction of secondary metabolites, the supernatant is not acidified/pH not changed.

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Recipes

1. King's B agar and broth
    Protease peptone 20 g/L
    Glycerol 20 g/L
    Anhydrous K_2HPO_4 1.5 g/L
    $MgSO_4 \cdot 7H_2O$, 6.09 ml of 1 M solution
    Agar (for solid medium only), 15 g/L
    Deionized H_2O, 1,000 ml
    Completely dissolve protease peptone, glycerol and anhydrous K_2HPO_4 in 500 ml of deionized H_2O and adjust the pH to 7.2. Make up the final volume to 994 ml and autoclave for 20 min. Separately, prepare 1 M solution of $MgSO_4 \cdot 7H_2O$ and autoclave it. Add 6.00 ml of this solution to the autoclaved medium for making up the final volume to 1,000 ml

Acknowledgments

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