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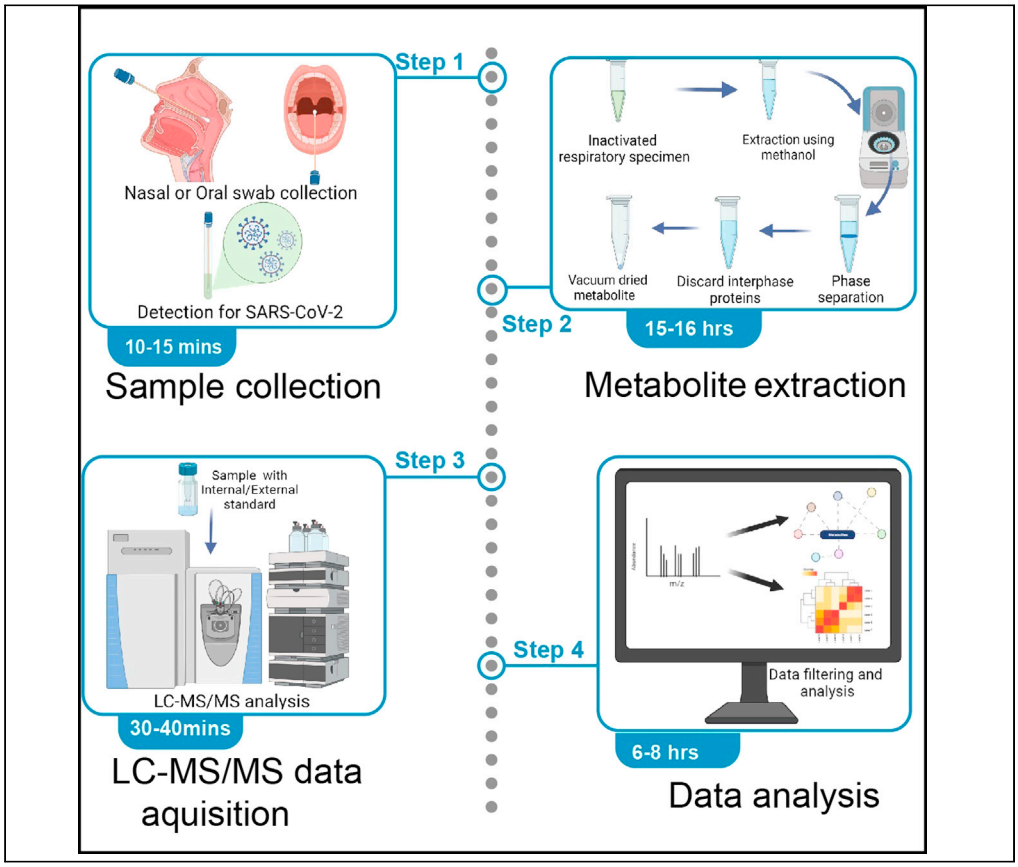
Protocol

Global metabolome profiling of COVID-19 respiratory specimen using high-resolution mass spectrometry (HRMS)



Here we describe a protocol for identifying metabolites in respiratory specimens of patients that are SARS-CoV-2 positive, SARS-CoV-2 negative, or H1N1 positive. This protocol provides step-by-step instructions on sample collection from patients, followed by metabolite extraction. We use ultra-high-pressure liquid chromatography (UHPLC) coupled with high-rResolution rass spectrometry (HRMS) for data acquisition and describe the steps for data analysis. The protocol was standardized with specific customization for SARS-CoV-2 containing respiratory specimens.

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Highlights

High-resolution mass spectrometry (HRMS)-based metabolomics of respiratory specimens

Heat inactivation, homogenization, and extraction of metabolites from patient samples

Peak picking and workflow for untargeted metabolomics data analysis

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Global metabolome profiling of COVID-19 respiratory specimen using high-resolution mass spectrometry (HRMS)

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SUMMARY

Here we describe a protocol for identifying metabolites in respiratory specimens of patients that are SARS-CoV-2 positive, SARS-CoV-2 negative, or H1N1 positive. This protocol provides step-by-step instructions on sample collection from patients, followed by metabolite extraction. We use ultra-high-pressure liquid chromatography (UHPLC) coupled with high-resolution mass spectrometry (HRMS) for data acquisition and describe the steps for data analysis. The protocol was standardized with specific customization for SARS-CoV-2-containing respiratory specimens.

For complete details on the use and execution of this protocol, please refer to Maras et al. (2021).

BEFORE YOU BEGIN

Note: This protocol has been used by our group (Maras et al., 2018, 2020, 2021; Bhat et al., 2020), and other groups (Weiss et al., 2016; Moreau et al., 2020) and (Castelli et al., 2021) for metabolome analysis.

Respiratory specimens were collected under the guidelines provided by the Centers for Disease Control and Prevention (Control and Prevention, 2020). The Viral Transport Media utilized is made of Hanks Balanced Salt Solution and contains a protective protein, antibiotics to control microbial and fungal contamination, and buffers to control the pH. Phenol red is used as a pH indicator. The medium also contains a cryoprotectant which helps in preserving the viruses if specimens are frozen for prolonged storage.

Nasopharyngeal specimen (NP) collection

Tilt the head of the patient 70° to the back. Gently insert and roll the swab with a flexible shaft (wire or plastic) through the nostril parallel to the palate (not upwards) until resistance is encountered or the distance is equivalent to that from the ear to the nostril of the patient, indicating contact with the nasopharynx. Leave the swab in place for a few seconds to absorb secretions. Slowly remove the swab while rotating it. Place swab, tip first, into the viral transport media containing tube provided.

Note: Specimens can be collected from both sides using the same swab, but it is not necessary to collect specimens from both sides if the swab is saturated with fluid from the first collection.

Oropharyngeal (OP) (throat) specimen collection

Insert swab into the posterior pharynx and tonsillar areas. Rub swab over both tonsillar pillars and posterior oropharynx and avoid touching the tongue, teeth, and gums. Place swab, tip first, into the viral transport media to make the respiratory specimen. Mix the NP and the OP specimen to form a respiratory specimen which can be used for RT-PCR based detection of viral presence and also could be used as a starting material for multi-omics analysis.

Note: The mixing of NP or OP is not mandatory and can be analyzed individually. An aliquot of the respiratory specimens was sent for SARS-CoV-2 detection, and the other was utilized for metabolomics analysis. Further, respiratory specimens can be stored in −80°C for a year. For better results, it should be used within 2 months for analysis

KEY RESOURCES TABLE

REREAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Acetonitrile for LCMS	J.T. Baker	Cat#14650359
Methanol for LCMS	Sigma-Aldrich	Cat#34860
Pierce™ Positive Ion Calibration Solution	Thermo Scientific	Cat#88323
Pierce™ Negative Ion Calibration Solution	Thermo Scientific	Cat#88324
LC-MS grade Formic acid	Merck	CAS#64-18-6
Milli-Q water (18 MU)	Thermo Scientific	Cat#W6-4
Metformin	Sigma-Aldrich	CAS#1115-70-4
Ethylmalonic acid	Sigma-Aldrich	CAS#601-75-2
Dihydrostreptomycin	Sigma-Aldrich	CAS#5490-27-7
Colchicine	Sigma-Aldrich	CAS#64-86-8
Imipramine	Sigma-Aldrich	CAS#113-52-0
Roxithromycin	Sigma-Aldrich	CAS#80214-83-1
Amiloride	Sigma-Aldrich	CAS#2016-88-8
Atropine	Sigma-Aldrich	CAS#51-55-8
2-aminoanthracene	Sigma-Aldrich	CAS#613-13-8
Prednisolone	Sigma-Aldrich	CAS#50-24-8
Dinoseb	Sigma-Aldrich	CAS#88-85-7
MCPA 2-methyl-4-chlorophenoxyacetic acid	Sigma-Aldrich	CAS#2436-73-9
Dimetridazole	Sigma-Aldrich	CAS#551-92-8
AMPA 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid	Sigma-Aldrich	Cat#A6816
Viral Transport Media	Himedia	Cat#MS2760A
Software and algorithms		
Compound Discoverer Software 3.1	Thermo Fisher Scientific	Compound Discoverer™ Software
MetaboAnalyst 4.0	Web server	https://www.metaboanalyst.ca/
Other		
Q Exactive Orbitrap Mass Spectrometer	Thermo Scientific	Cat#IQLAAEGAAPFALGMBDK
Hypersil GOLD™ C18 Selectivity HPLC Columns	Thermo Scientific	Cat#25003102130
200µL pipette tips, Sterile grade	Eppendorf® Combitips advanced	Z762997
9mm Target DP 300µL HPLC Vial (Polypropylene)	Thermo Scientific	C4000-11
9mm Autosampler Vial Screw Thread Caps (Blue), PTFE/White Silicone Septum	Thermo Scientific	C5000-54B
Benchtop centrifuge	Eppendorf	Cat#5409000012
Sonicator	Helix Biosciences	I.T NO.#HBSNII-92
Vortex	Sigma-Aldrich	Z258423
Vacuum evaporator	Genevac	Cat#75871-454

MATERIALS AND EQUIPMENT

Internal standards for untargeted analysis		
Reagent	Concentration	Amount
Dinoseb	5 μg/mL	25 μg
MCPA 2-methyl-4-chlorophenoxyacetic acid	5 μg/mL	25 μg
Dimetridazole	5 μg/mL	25 μg
AMPA 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid	5 μg/mL	25 μg
Milli-Q water	n/a	Make-up volume
Total		5 mL

External standard for untargeted analysis		
Reagent	Concentration	Amount
Dihydrostreptomycin	20 μg/mL	100 μg
Colchicine	0.5 μg/mL	2.5 μg
Imipramine	0.5 μg/mL	2.5 μg
Roxithromycin	20 μg/mL	100 μg
Amiloride	10 μg/mL	50 μg
Atropine	1 μg/mL	5 μg
2-aminoanthracene	1 μg/mL	5 μg
Prednisolone	1 μg/mL	5 μg
Metformin	1 μg/mL	5 μg
Ethylmalonic acid	3 μg/mL	15 μg
Milli-Q water		Make-up volume
Total		5 mL

Solvent A for untargeted analysis		
Reagent	Concentration	Amount (500 mL)
Formic Acid	0.1%	1 mL
Milli-Q water		499 mL

Note: Always prepare fresh Solvent A. To prevent contamination of any particulate matter filter the solvent with 0.45 μm filter. Then place the bottle in an ultrasonic bath for 10 min to degas the solvent. To avoid shift in the retention time, the same sample set should be run within 2–3 days with the fresh mobile phase solvent.

⚠ CRITICAL: Formic acid is highly caustic and flammable liquid, with a strong pungent odor. It can cause damage to skin, eyes, and mucosal surfaces. If contact occurs, the person should flush the affected areas immediately with plenty of water, followed by washing with soap and water. Wear protective gloves, laboratory coat, eye and face protection while working with this chemical

Solvent B for untargeted analysis		
Reagent	Concentration	Amount (500 mL)
Acetonitrile for LC-MS	100%	500 mL

Note: Always prepare fresh Solvent B. To prevent contamination of any particulate matter filter the solvent with 0.45 μm filter. Mix the solvent gently. Then place the bottle in an ultrasonic

Table 1. LC-MS parameter settings

Ionization	Electrospray ionization
MS1 and MS2 mass ranges	m/z 150–m/z 2,000
Sheath gas flow rate	54
Auxiliary gas flow rate	13
Resolution	70,000 m/Δ m
Collision gas	Nitrogen
Collision energy (positive mode/negative mode)	+40/-42 eV
Collision energy spread	15 eV
Ion source gas 1 (air; positive mode/negative mode)	40/50 psi
Ion source gas 2 (air; positive mode/negative mode)	80/50 psi
Curtain gas (nitrogen)	30 psi
Ion source temperature (positive mode/negative mode)	250°C/300°C
Ion spray voltage floating (positive mode/negative mode)	5500–4500 V
Declustering potential (positive mode /negative mode)	80/−80 eV

bath for 10 min to degas the solvent. To avoid shift in the retention time, the same sample set should be run within 2–3 days with the fresh mobile phase solvent.

⚠ CRITICAL: Acetonitrile is a colorless volatile liquid with an aromatic odor, highly flammable and hazardous if inhaled or absorbed by skin. One should use a fume hood to minimize exposure to this substance. Wear protective clothing to avoid skin or eye contact, inhalation or ingestion. A long-sleeved laboratory coat or gown, rubber gloves, safety goggles and a face mask as a minimum standard.

- LC-MS setup for untargeted analysis:
- Metabolomic analysis is performed on the Thermo Scientific™ UHPLC system combined with Q Exactive Orbitrap Mass Spectrometers.
- Samples are analyzed in single runs for positive and negative modes using the parameters shown in Table 1.
- Hypersil GOLD™ C18 Selectivity HPLC Column is used as a stationary phase to separate respiratory metabolites. Column Specifications are mentioned in Table 2.
- The mobile phases consist of (A) Acetonitrile with 1% formic acid and (B) Milli-Q-water with 0.1% formic acid, used for gradient elution (Table 3).

Table 2. Column formats

Column format	Analytical column
For Use With	LC/MS
Max. Pressure	5800 psi (400 bar)
pH	1 to 11
Surface Area	220 m^2/g
USP Type	L1
Product Line	Hypersil GOLD
Carbon Load	11%
Endcapped	Yes
Diameter (Metric)	2.1 mm
Length (Metric)	100 mm
Particle Size	3 µm
Pore Size	175 Å
Temperature	60°C
Stationary Phase	C18 Selectivity
Packing Material	Spherical, Fully Porous, Ultrapure Silica
Column Type	Reversed Phase

Table 3. LC-MS gradient for untargeted metabolomics

S. No.	Time (min)	Flow (mL/min)	% B	Curve
1	0	0.500	5.0	5
2	1	0.500	5.0	5
3	17	0.500	99.0	5
4	21	0.500	99.0	5
5	22	0.500	5.0	5
6	25	Stop Run		

Note: Overall run cycle time per sample is 25 min.

STEP-BY-STEP METHOD DETAILS

An outline of the procedures is described in Figure 1. A total of 500 µL respiratory specimens was heat-inactivated using heat block at 95°C for 15 min in a BSL3 lab. Heat inactivated respiratory specimens were subjected to homogenization by probe sonicator (steps 1–6). Respiratory metabolites were extracted by organic phase extraction method (steps 7–18) for untargeted metabolomics using LCMS (steps 19–20). The obtained data are analyzed using the Compound discoverer version 3.1 (steps 21–31).

Heat inactivation of virus

Timing: 15 min/sample

This step is crucial to inactivate the virus as a safety measure to mitigate the risk of infection during sample preparation.

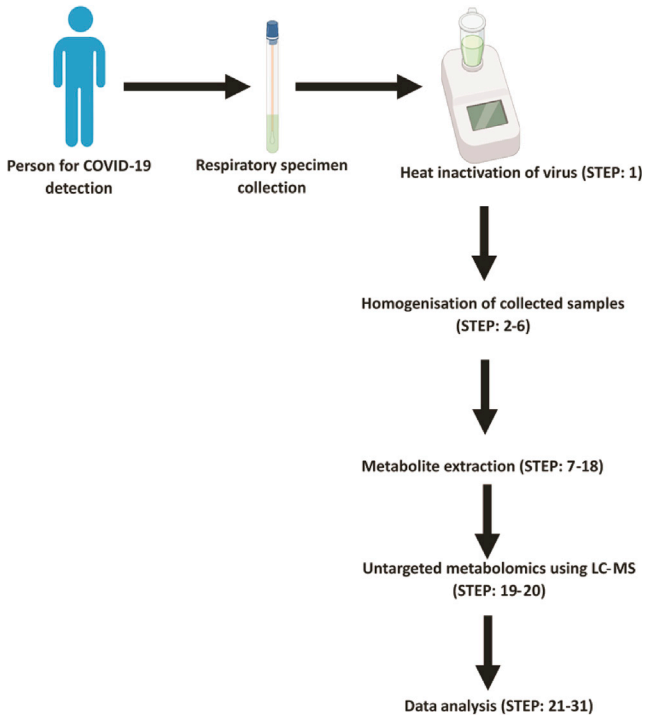


Figure 1. Showing an outline of the respiratory specimen collection, preparation, and data analysis

1. The collected respiratory specimens are placed on a heating block at 95°C for 15 min (Batejat et al., 2021) to inactivate the virus.

Pause point: Respiratory specimen can be stored in −80°C upto 4 weeks

Homogenization of respiratory specimen

Timing: 5–7 min/sample

Respiratory specimens may contain host cells, mucus, bacterial and viral components. Thus, it is essential to homogenize the samples.

⚠ CRITICAL: The high frequency sound emitted by the sonicator can damage hearing. Therefore place the sample in a noise isolating chamber and always close the door while operating. Do not grasp an activated horn or touch the tip of a vibrating probe. It can cause severe burns and tissue damage.

2. Take 100 µL of respiratory specimen in a new microcentrifuge tube (MCT) and keep it on ice during homogenization.
3. Place the sample in a noise isolating chamber and submerge the sonicator probe into the sample.

Note: The probe should not touch the walls of MCT as it will break the tube and destroy the sample.

4. Close the door of the chamber after correctly placing the sample tube.
5. Run the program at Power 20%, Run time 5 min (Cycle - 10 s ON, 10 s OFF) and temperature 22°C.
6. Remove and clean the probe with ethanol.

Note: Keep the sample on ice during all the homogenization steps step 2–6.

Pause point: Respiratory specimen can be stored in −80°C upto 4 weeks

Organic phase metabolite extraction

Timing: ~15 h/sample

To reduce the complexity of the sample, proteins must be removed. Hence, organic phase extraction will remove the proteins through precipitation and dissolve the metabolites in solution.

7. Add 100 uL of homogenized respiratory specimen to 400 uL of 100% chilled methanol.
8. Vortex it for 10 s.
9. Keep it in −20°C for 10–12 h.
10. Centrifuge the sample at 18,000*g* for 10 min.
11. Take the supernatant into a new tube and discard the pellet.
12. Freeze-dry the sample completely using a vacuum concentrator.
13. Dissolve the sample in 105 uL of solvent (5% ACN (Acetonitrile), 5% Internal Standard, 95% water).
14. Vortex the sample for 10 s.
15. Pipette 80 uL of sample solution into HPLC tube.
16. Add the remaining 25 µL in a different tube to prepare a pooled quality control sample (In this tube all the samples prepared for run are pooled together).

Note: QC samples are required to correct the small levels of variation within samples from the same group, to quantitatively measure technical reproducibility and to integrate data from different analytical experiments. The peak features exceeding 30% coefficient of variation (CV) values and less than 0.7 r2 for Coefficient of correlation in QC samples are excluded.

17. Make a dilution of the Pool solution.

Sample – solvent ratio	Sample%	Solvent%
1:1	100% sample	
1:2	50% sample	50% solvent
1:4	25% sample	75% solvent
1:8	12.5% sample	87.5% solvent

18. Make a blank with 100% ACN.

Sample run and analysis

Timing: 30–40 min/sample

This step provides you MS.raw file for each sample for further analysis.

19. Lay down the sample sequence as exemplified in Figure 2.

	Sample Type	File Name	Sample ID	Path	Inst Meth	Position	Inj Vol	Comment
▶ 1	QC	POOL 1:8 DILUTION	POOL 1:8 DILUTION	C:\Xcalibur\Data		A1	*	
2	QC	POOL 1:8 DILUTION	POOL 1:8 DILUTION	C:\Xcalibur\Data		A1	*	
3	QC	POOL 1:8 DILUTION	POOL 1:8 DILUTION	C:\Xcalibur\Data		A1	*	
4	Blank	BLANK01	BLANK01	C:\Xcalibur\Data		A2	*	
5	QC	POOL 1:4 DILUTION	POOL 1:4 DILUTION	C:\Xcalibur\Data		A3	*	
6	QC	POOL 1:4 DILUTION	POOL 1:4 DILUTION	C:\Xcalibur\Data		A3	*	
7	QC	POOL 1:4 DILUTION	POOL 1:4 DILUTION	C:\Xcalibur\Data		A3	*	
8	Blank	BLANK02	BLANK02	C:\Xcalibur\Data		A2	*	
9	QC	POOL 1:2 DILUTION	POOL 1:2 DILUTION	C:\Xcalibur\Data		A4	*	
10	QC	POOL 1:2 DILUTION	POOL 1:2 DILUTION	C:\Xcalibur\Data		A4	*	
11	QC	POOL 1:2 DILUTION	POOL 1:2 DILUTION	C:\Xcalibur\Data		A4	*	
12	Blank	BLANK03	BLANK03	C:\Xcalibur\Data		A2	*	
13	QC	POOL 1:2 DILUTION	POOL 1:2 DILUTION	C:\Xcalibur\Data		A5	*	
14	QC	POOL 1:2 DILUTION	POOL 1:2 DILUTION	C:\Xcalibur\Data		A5	*	
15	QC	POOL 1:2 DILUTION	POOL 1:2 DILUTION	C:\Xcalibur\Data		A5	*	
16	Blank	BLANK04	BLANK04	C:\Xcalibur\Data		A2	*	
17	QC	POOL 1:1 DILUTION	POOL 1:1 DILUTION	C:\Xcalibur\Data		A6	*	
18	QC	POOL 1:1 DILUTION	POOL 1:1 DILUTION	C:\Xcalibur\Data		A6	*	
19	QC	POOL 1:1 DILUTION	POOL 1:1 DILUTION	C:\Xcalibur\Data		A6	*	
20	Blank	BLANK05	BLANK05	C:\Xcalibur\Data		A2	*	
21	Unkno...	SAMPLE_1	SAMPLE_1	C:\Xcalibur\Data		A7	*	
22	Unkno...	SAMPLE_2	SAMPLE_1	C:\Xcalibur\Data		A8	*	
23	Unkno...	SAMPLE_3	SAMPLE_1	C:\Xcalibur\Data		B1	*	
24	Unkno...	SAMPLE_4	SAMPLE_1	C:\Xcalibur\Data		B2	*	
25	Unkno...	SAMPLE_5	SAMPLE_1	C:\Xcalibur\Data		B3	*	
26	Blank	BLANK06	BLANK06	C:\Xcalibur\Data		A2	*	
27	QC	POOL 1:1 DILUTION	POOL 1:1 DILUTION	C:\Xcalibur\Data		A6	*	
28	Unkno...	SAMPLE_6	SAMPLE_6	C:\Xcalibur\Data		B4	*	
*								

Figure 2. Showing an exemplary sample sequence list

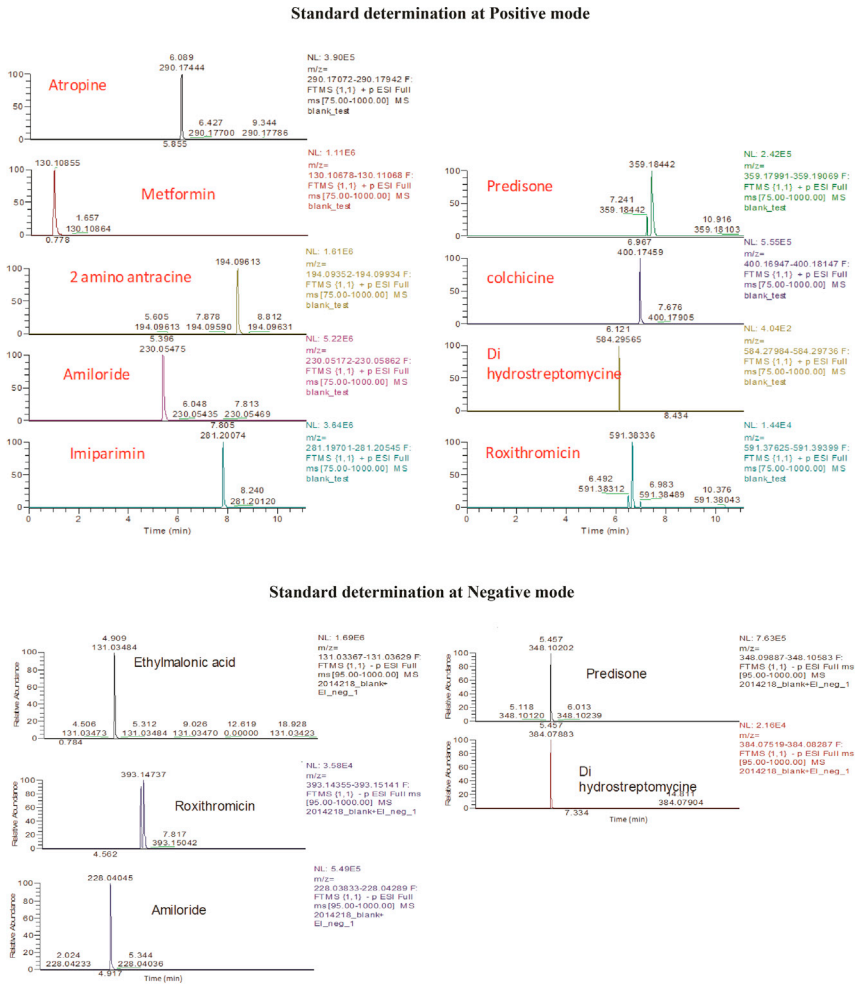


Figure 3. Showing mass spectral peak of standard metabolites

20. Set the run with LC-MS parameters described in Table 1 (Boudah et al., 2014) and run gradient is described in Table 3. Further details are provided in Table S1.

Peak picking and software analysis

Timing: 6–8 h/sample

MS.raw file is mapped on different metabolome databases for metabolite annotation that is exported in excel sheet for statistical analysis in order to interpret the data.

21. Standard determination at both positive and negative mode as shown in Figure 3.

Data analysis using Compound discoverer ver. 3.1:

Compound Discoverer is a unique small molecule structure recognition software. It uses accurate mass data, isotope pattern matching, fragment matching, and mass spectral library search to identify the structure of small molecules. It is a qualitative data processing application that can process accurate mass spectra of the entire Thermo Scientific high-resolution mass spectrometer product

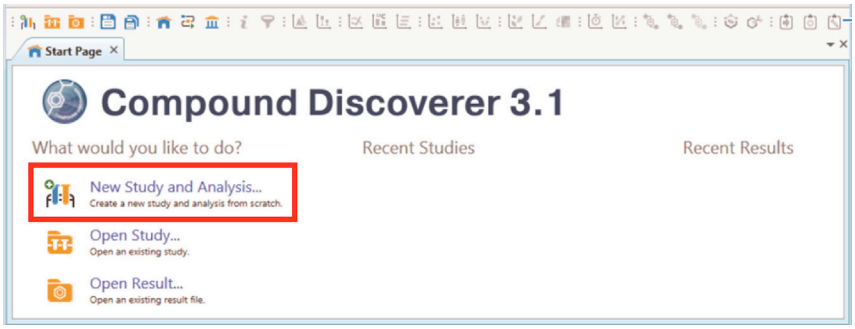


Figure 4. Graphical representation of step 22
Compound discoverer 3.1 start page for new study and analysis.

line. Compound Discoverer provides a set of processing tools called Nodes, which can be combined into a processing workflow in many different ways according to the type of experiment you perform and the questions you must answer. These workflows can be saved and reused as templates. Additionally, users can even design and write their own nodes for use in Compound Discoverer.

22. Open the Compound discoverer ver. 3.1 application. Click ''New Study and Analysis'' (in red borders; Figure 4).
23. The New Study and Analysis Wizard opens. Click ''Next'' (indicated by the red arrow in Figure 5).
24. Define the study type, name of study and storage location (underlined in red). Study template file and workflow can also be optionally selected. Then click ''Next'' (red arrow; Figure 6).
25. Click ''Add Files'' on the Input File Selection page of the wizard to select the sample data files. The file names of the selected files appear in the Files box. And then click ''Next'' (indicated by the red arrow in Figure 7).
26. In the Input File Characterization dialog box, define the Sample Type for blanks, samples, and pools and click on ''Finish'' (Figure 8).

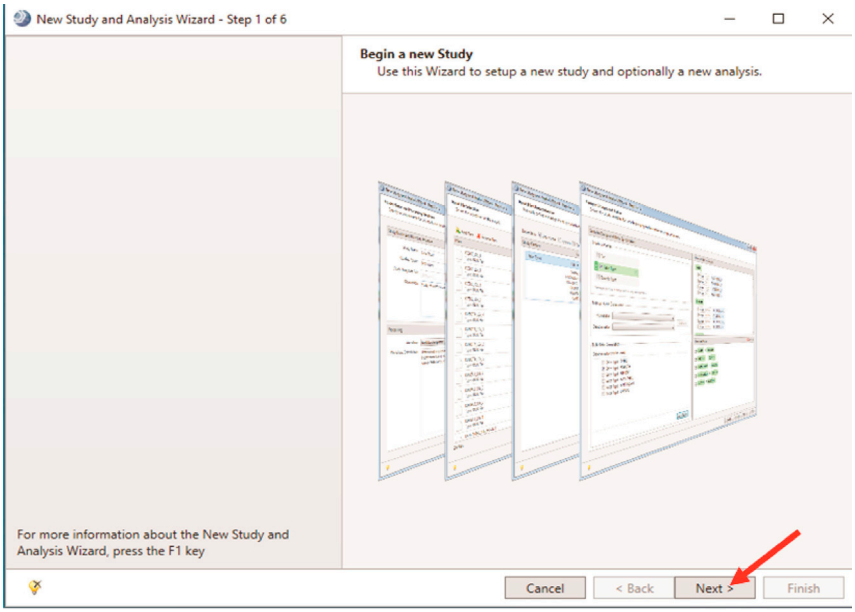


Figure 5. Graphical representation of step 23
Showing New Study and Analysis Wizard.

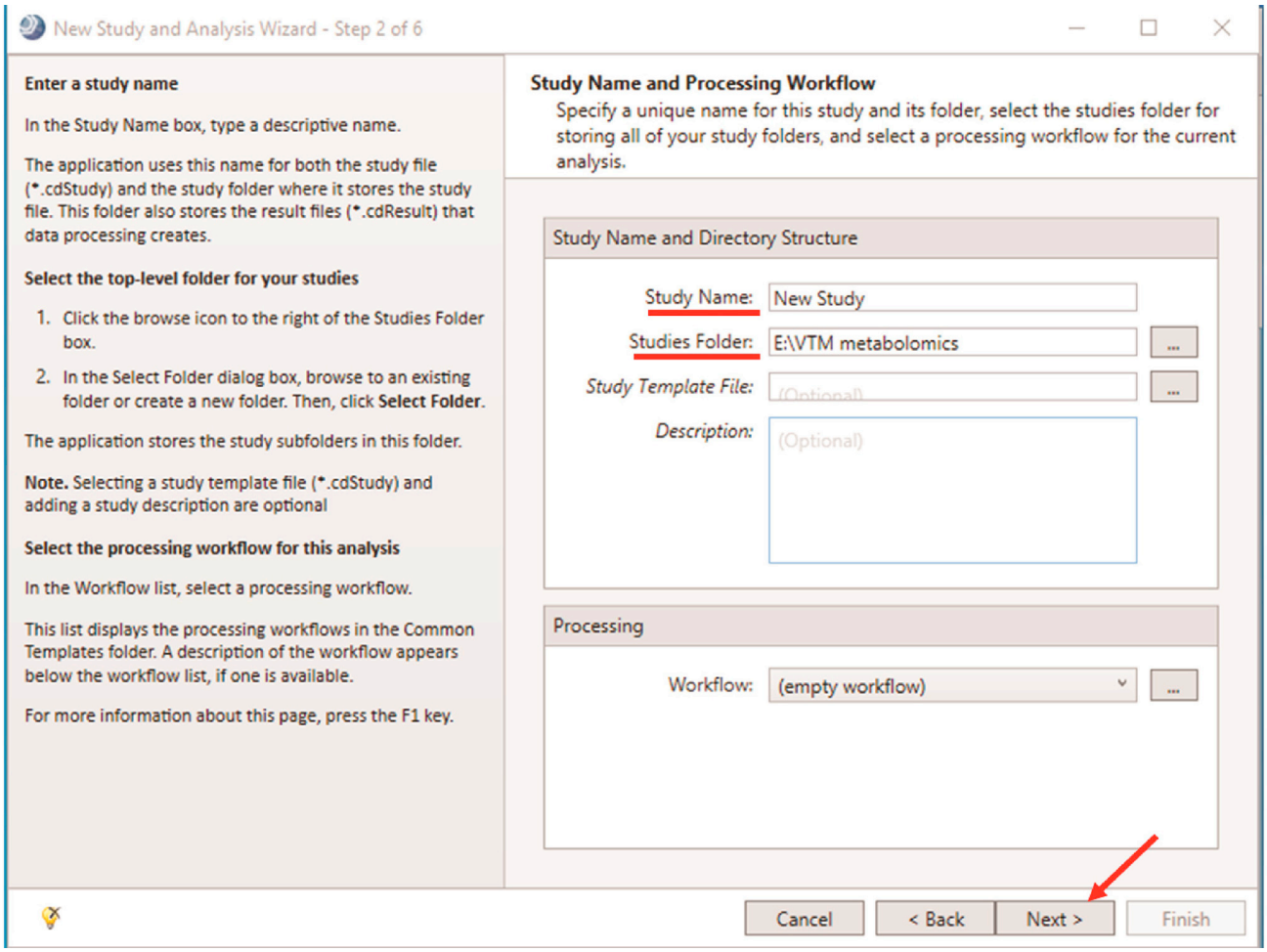


Figure 6. Graphical representation of step 24
Name the study and describe the path of study.

27. Select the metabolomics workflow in the Workflows tab appear in the set of tabbed pages.

    Note: The workflow is utilized by Maras et al. (2021) shown below in Figure 9: each node is explained in user manual provided at https://assets.thermofisher.com ⟩ CMD ⟩ manuals

28. Name the result file in the Analysis pane and click Run to submit the analysis to the job queue (Figure 10).
29. When the job is done, a result layout file will appear (Figure 11), export the result file in ".xlsx" format.

    Note: Peak picking based on "mass + retention time, mass + retention time + spectral match and mass alone."

30. In the excel file obtained, remove the duplicates (red borders) based on average area intensity of the samples (Figure 12).
31. Create a comma delimited file of annotated metabolites along with their sample wise area intensity and do statistical analysis using software such as Metaboanalyst webserver (Chong et al., 2019) (Figure 13).

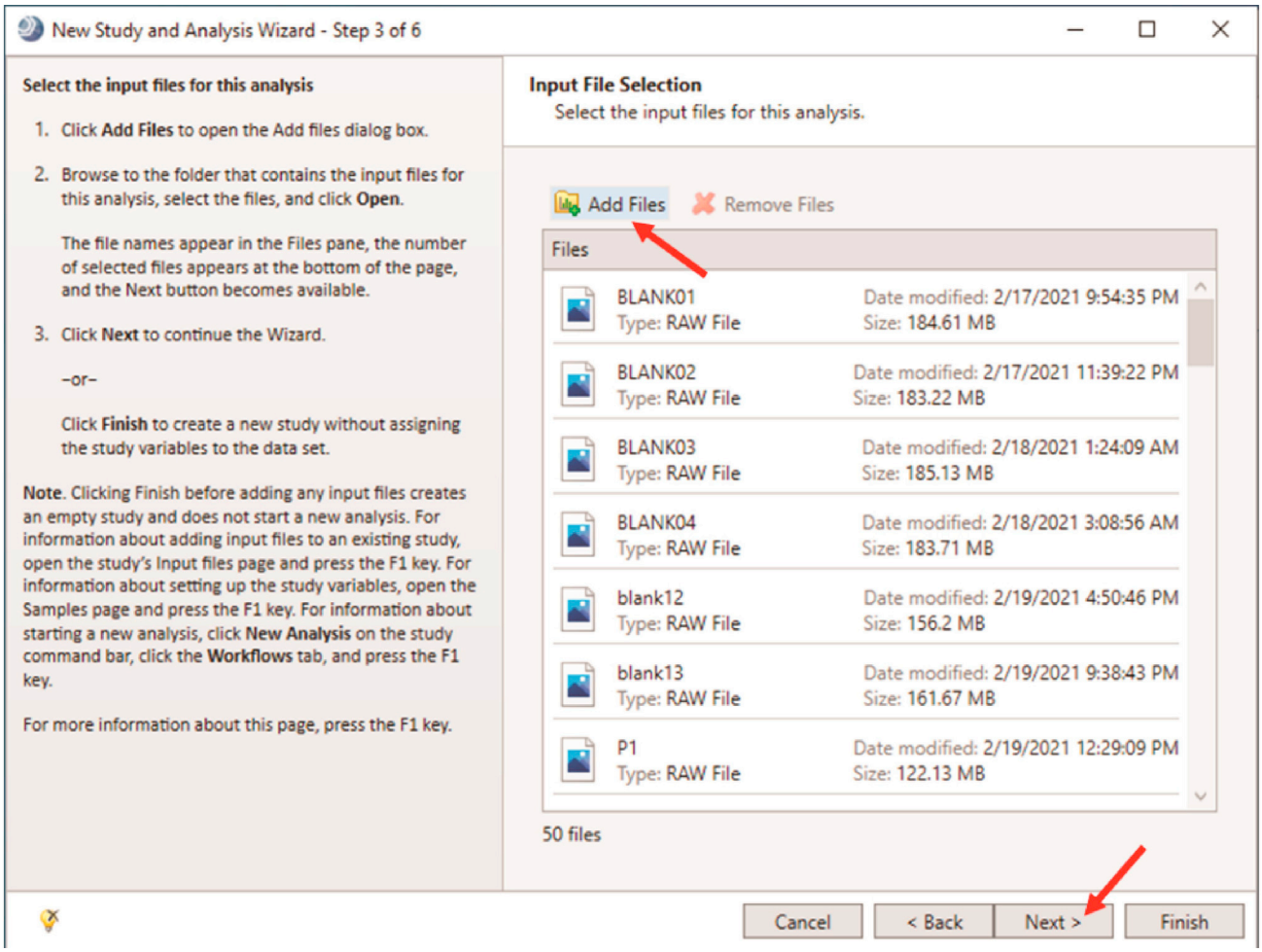
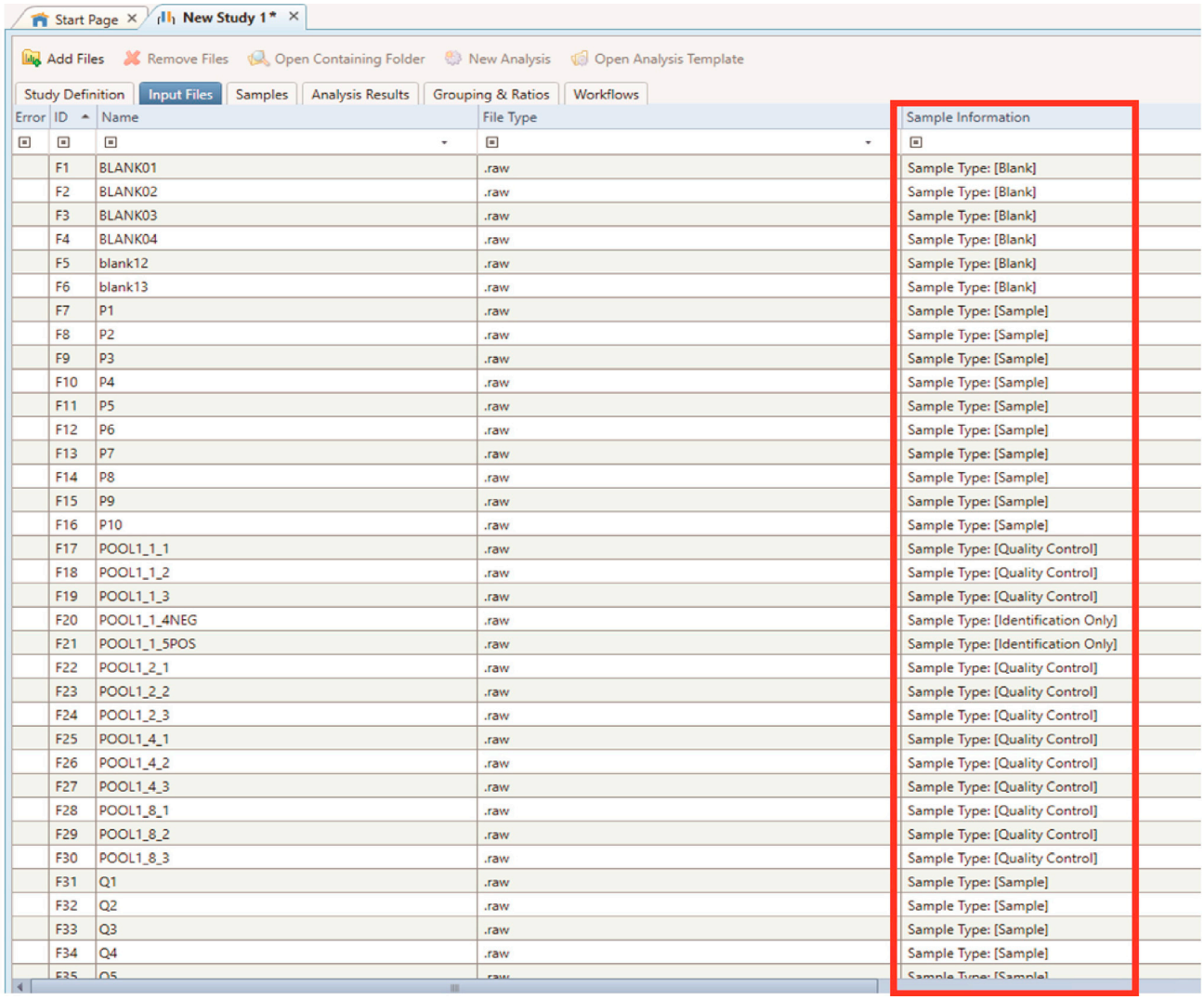


Figure 7. Graphical representation of step 25, showing Input File Selection Dialogue Box

EXPECTED OUTCOMES

Untargeted metabolomics analysis of the respiratory specimen could yield close to 20 thousand to 40 thousand ions depending on the machine sensitivity and specificity. Those ions which qualify analytical quality determination are subjected to annotation using Spectral Database and database of pure compound which document RT and intact mass for the same in the compound discoverer software. The compound discoverer software returns annotation for the probable ions which need to be checked individually and processed for statistical analysis for the study group. Statistical analysis of the metabolites within the VTM showed 106 metabolites that were significantly dysregulated in COVID-19 positive patients. Metabolites like N-acetylserotonin (C00978) and azelaic acid (C08261) had the highest mean decrease in accuracy and showed a combined diagnostic efficiency of 0.987 (0.98–1) for SARS-CoV-2 positive segregation from negatives. Pathway analysis revealed significant increase in pathways linked to biosynthesis of unsaturated fatty acids, glycerophospholipid metabolism, ubiquinone/terpenoid-quinone biosynthesis, aminoacyl-tRNA biosynthesis and amino acid metabolism including phenylalanine, tyrosine and tryptophan biosynthesis whereas, pathways linked to thiamine metabolism, one carbon pool by folate, vitamin B6 metabolism, riboflavin metabolism and steroid biosynthesis were decreased (Figure 13). Therefore, SARS-CoV-2 infection tends to change the metabolic phenotype of respiratory specimens.



Error	ID	Name	File Type	Sample Information
	F1	BLANK01	.raw	Sample Type: [Blank]
	F2	BLANK02	.raw	Sample Type: [Blank]
	F3	BLANK03	.raw	Sample Type: [Blank]
	F4	BLANK04	.raw	Sample Type: [Blank]
	F5	blank12	.raw	Sample Type: [Blank]
	F6	blank13	.raw	Sample Type: [Blank]
	F7	P1	.raw	Sample Type: [Sample]
	F8	P2	.raw	Sample Type: [Sample]
	F9	P3	.raw	Sample Type: [Sample]
	F10	P4	.raw	Sample Type: [Sample]
	F11	P5	.raw	Sample Type: [Sample]
	F12	P6	.raw	Sample Type: [Sample]
	F13	P7	.raw	Sample Type: [Sample]
	F14	P8	.raw	Sample Type: [Sample]
	F15	P9	.raw	Sample Type: [Sample]
	F16	P10	.raw	Sample Type: [Sample]
	F17	POOL1_1_1	.raw	Sample Type: [Quality Control]
	F18	POOL1_1_2	.raw	Sample Type: [Quality Control]
	F19	POOL1_1_3	.raw	Sample Type: [Quality Control]
	F20	POOL1_1_4NEG	.raw	Sample Type: [Identification Only]
	F21	POOL1_1_5POS	.raw	Sample Type: [Identification Only]
	F22	POOL1_2_1	.raw	Sample Type: [Quality Control]
	F23	POOL1_2_2	.raw	Sample Type: [Quality Control]
	F24	POOL1_2_3	.raw	Sample Type: [Quality Control]
	F25	POOL1_4_1	.raw	Sample Type: [Quality Control]
	F26	POOL1_4_2	.raw	Sample Type: [Quality Control]
	F27	POOL1_4_3	.raw	Sample Type: [Quality Control]
	F28	POOL1_8_1	.raw	Sample Type: [Quality Control]
	F29	POOL1_8_2	.raw	Sample Type: [Quality Control]
	F30	POOL1_8_3	.raw	Sample Type: [Quality Control]
	F31	Q1	.raw	Sample Type: [Sample]
	F32	Q2	.raw	Sample Type: [Sample]
	F33	Q3	.raw	Sample Type: [Sample]
	F34	Q4	.raw	Sample Type: [Sample]
	F35	Q5	.raw	Sample Type: [Sample]

Figure 8. Graphical representation of step 26
In the Input File Characterization dialog box define the sample type.

LIMITATIONS

The precise determination of the actual quantitate of the metabolites could not be determined by this method. In turn this method returns quantity in arbitrary units. Further this method cannot distinguish between host and virus or microbe linked metabolites nor could assess the flux of these metabolites.

TROUBLESHOOTING

Problem 1

Leaks in the system (step 20).

Potential solution

Inspect all the fittings for leaks. Tighten any loose fittings, without over tightening them as this may cause damage to the fitting's threads and cause leaks. Replace the fitting and ferrule if they may be damaged.

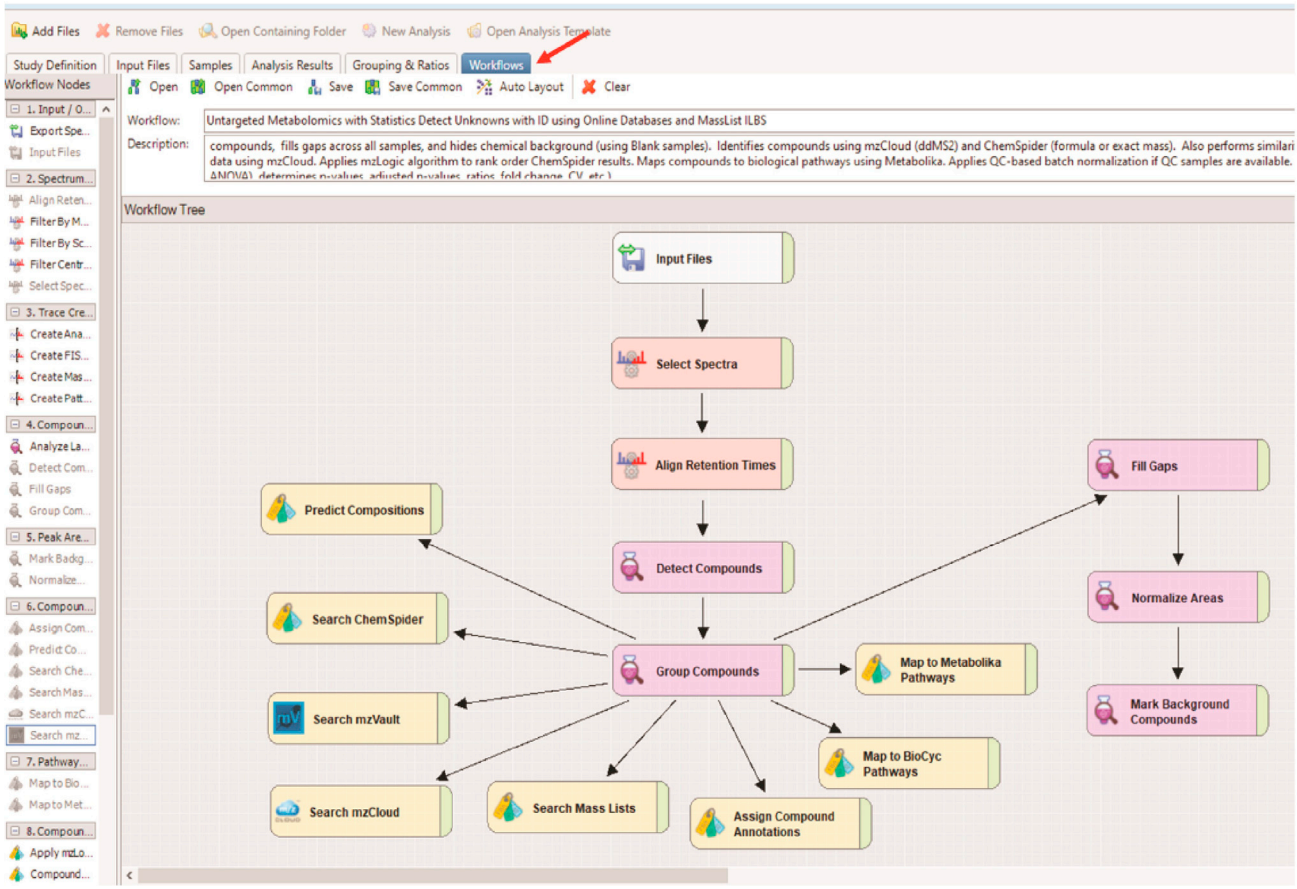


Figure 9. Graphical representation of step 27, showing untargeted metabolome workflow

Problem 2

No peaks/very small peaks (step 20).

Potential solution

First of all, check the lamp is on and cables are well connected. Next make sure if the flow is normal and automatic sampler vials have sufficient liquid and no air bubbles in the sample. If the problem still persists, then evaluate the system performance with fresh standards to confirm if sample is the source of problem.

Problem 3

If the pressure is higher than normal (step 20).

Potential solution

Remove the guard and analytical column. Check the pressure, then isolate the cause by systematically eliminating system components, starting with detector, then in-line filter, and working back to pump. Replace filter in pump if present. Replace guard column if necessary. If the analytical column is obstructed, reverse and flush the column while disconnected from the detector. If the problem persists, the column may be clogged with strongly retained contaminants. Therefore, change the column.

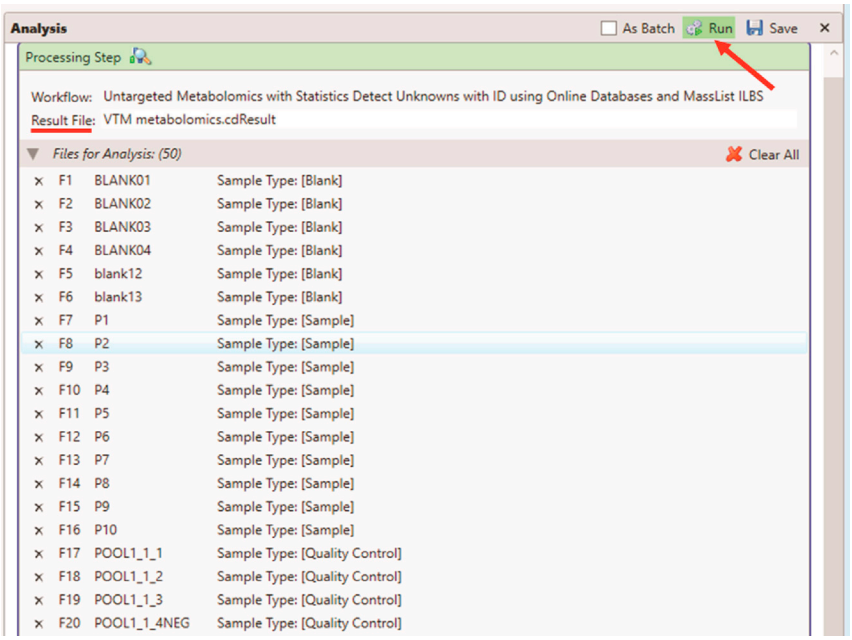


Figure 10. Graphical representation of step 28, name the result file and click 'Run'

Problem 4

If there is a shift in Retention Times (step 20).

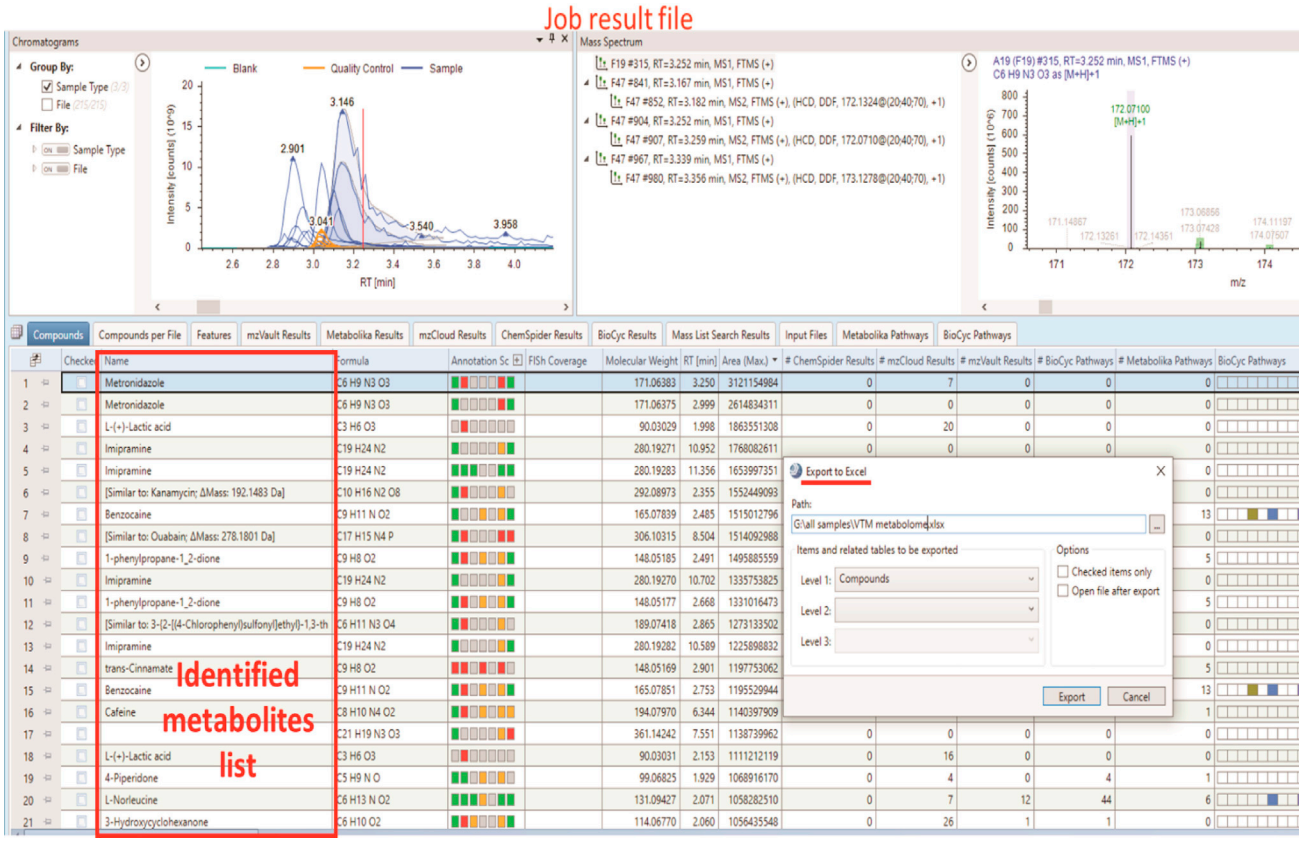
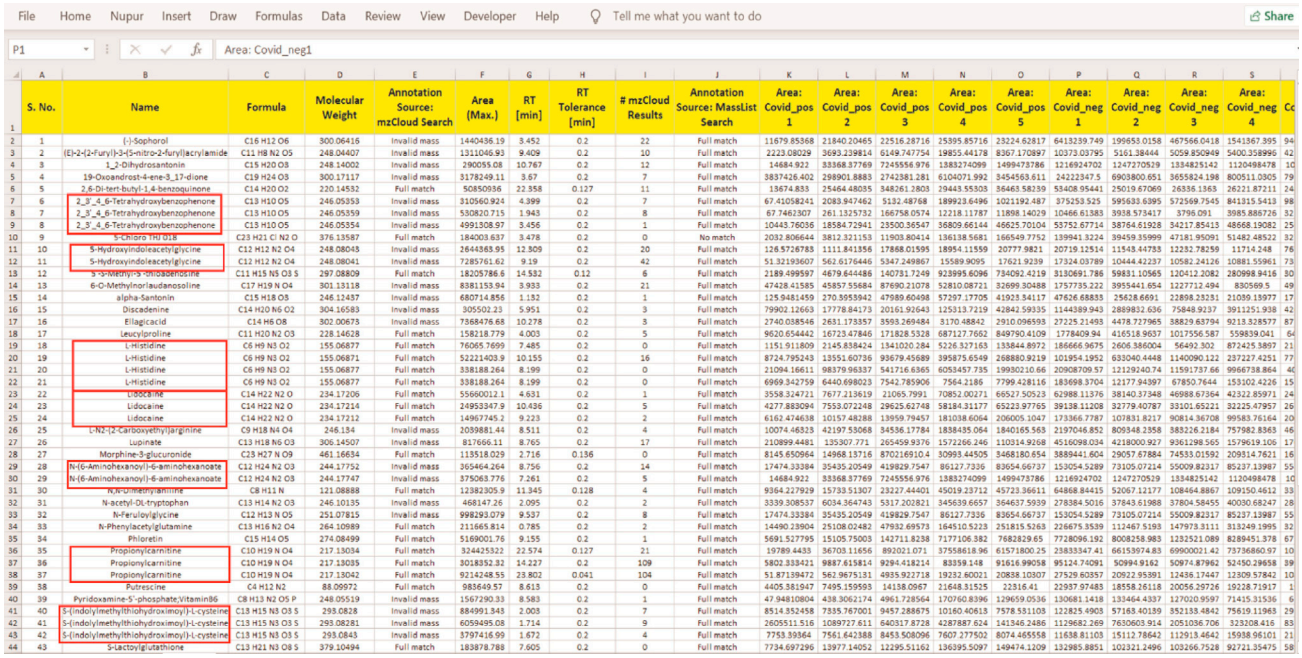


Figure 11. Showing result layout file (step 29)



S. No.	Name	Formula	Molecular Weight	Annotation Source: mzCloud Search	Area (Max.)	RT [min]	RT Tolerance [min]	# mzCloud Results	Annotation Source: MassList Search	Area: Covid_pos 1	Area: Covid_pos 2	Area: Covid_pos 3	Area: Covid_pos 4	Area: Covid_pos 5	Area: Covid_neg 1	Area: Covid_neg 2	Area: Covid_neg 3	Area: Covid_neg 4
1	(-)-Sophorol	C16 H12 O6	300.06416	Invalid mass	1440436.19	3.452	0.2	22	Full match	11679.85368	21840.20465	22516.28716	25395.85716	23224.62817	6413239.749	199653.0158	467566.0418	1541367.395
2	(E)-2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide	C11 H8 N2 O5	248.04407	Invalid mass	1311046.93	9.409	0.2	10	Full match	2223.08029	3693.239814	6149.747754	19855.44178	8367.170897	10373.03795	5161.38444	5059.850949	5400.358996
3	1_2-Dihydrosantonin	C15 H20 O3	248.14002	Invalid mass	290055.08	10.767	0.2	12	Full match	14684.922	33368.37769	7245556.976	1383274099	1499473786	1216924702	1247270529	1334825142	1120498478
4	19-Oxoandrost-4-ene-3_17-dione	C19 H24 O3	300.17117	Invalid mass	3178249.11	3.67	0.2	7	Full match	3837426.402	298901.8883	2742381.281	6104071.992	3454563.611	24222347.5	6903800.651	3655824.198	8005113.030
5	2_6-Di-tert-butyl-1_4-benzoquinone	C14 H20 O2	220.14532	Full match	50850936	22.358	0.127	11	Full match	13674.833	25464.48035	348261.2803	29443.55305	56463.58239	53408.95441	25019.67069	26336.1363	26221.87211
6	2_3'_4_6-Tetrahydroxybenzophenone	C13 H10 O5	246.05353	Invalid mass	310560.924	4.399	0.2	7	Full match	67.41058241	2083.947462	5132.48768	189923.6496	1021192.487	375253.525	595633.6395	572569.7545	841315.5413
7	2_3'_4_6-Tetrahydroxybenzophenone	C13 H10 O5	246.05359	Invalid mass	530820.715	1.943	0.2	8	Full match	67.7462307	261.1325732	166758.0574	12218.11787	11898.14029	10466.61383	3938.573417	3796.091	3985.888726
8	2_3'_4_6-Tetrahydroxybenzophenone	C13 H10 O5	246.05354	Invalid mass	4991308.97	3.456	0.2	1	Full match	10443.76036	18584.72941	23500.36547	36809.66144	46625.70104	53752.67714	38764.61928	34217.85413	48668.19082
9	5-Chloro TRI 018	C23 H21 Cl N2 O	376.13587	Full match	184003.637	3.478	0.2	0	No match	2032.806644	3812.321153	11903.80414	136138.5681	166549.7752	139941.3224	39459.35999	47181.95091	51482.48522
10	5-Hydroxyindoleacetylglycine	C12 H12 N2 O4	248.08043	Invalid mass	2644363.95	12.309	0.2	20	Full match	126.5726783	1111.841356	17868.01595	18954.11559	20777.9821	20719.12514	11543.44733	12232.78259	11714.248
11	5-Hydroxyindoleacetylglycine	C12 H12 N2 O4	248.08041	Invalid mass	7285761.62	9.19	0.2	42	Full match	51.32193607	562.6176446	5347.249867	15589.9095	17621.9239	17324.03789	10444.42237	10582.24126	10881.55961
12	5'-S-Methyl-5'-thioadenosine	C11 H15 N5 O3 S	297.08809	Full match	18205786.6	14.532	0.12	6	Full match	2189.499597	4679.644486	140731.7249	923995.6096	734092.4219	3130691.786	59831.10565	120412.2082	280998.9416
13	6-O-Methylnorlaudanosoline	C17 H19 N O4	301.13118	Invalid mass	8381153.94	3.933	0.2	21	Full match	47428.41585	45857.55684	87690.21078	52810.08721	32699.30488	1757735.222	3955441.654	1227712.494	830569.5
14	alpha-Santonin	C15 H18 O3	246.12457	Invalid mass	680714.856	1.132	0.2	1	Full match	125.9481459	270.3955942	47989.60498	57297.17705	41923.34117	47626.68833	25628.6691	22898.23251	21039.13977
15	Discadenine	C14 H20 N6 O2	304.16583	Invalid mass	305502.23	5.951	0.2	3	Full match	79902.12663	17778.84173	20161.92643	125313.7219	42842.59335	1144389.943	2889832.636	75848.9237	3911251.938
16	Ellagicacid	C14 H6 O8	302.00673	Invalid mass	7368476.68	10.278	0.2	3	Full match	2740.038546	2631.173357	3593.269484	3170.48842	2910.096593	27225.21493	4478.727965	38829.63794	9213.328577
17	Leucylproline	C11 H20 N2 O3	228.14628	Full match	158218.779	4.003	0.2	5	Full match	9620.654442	16723.47846	171828.5328	687127.7662	849790.4109	1778409.94	416518.9637	1017556.587	559839.041
18	L-Histidine	C6 H9 N3 O2	155.06877	Full match	76065.7699	7.485	0.2	0	Full match	1151.911809	2145.838424	1341020.284	5226.327163	133844.8972	186666.9675	2606.386004	56492.302	872425.3897
19	L-Histidine	C6 H9 N3 O2	155.06871	Full match	522214O3.9	10.155	0.2	16	Full match	8724.795243	13551.60736	93679.45689	395875.6549	268880.9219	1019541.952	633040.4448	1140090.122	237227.4251
20	L-Histidine	C6 H9 N3 O2	155.06877	Full match	338188.264	8.199	0.2	0	Full match	21094.16611	98379.96337	541716.6365	6054357.735	1998302.166	20908709.57	1212924.74	11591737.66	9966738.864
21	L-Histidine	C6 H9 N3 O2	155.06877	Full match	338188.264	8.199	0.2	0	Full match	6969.342759	6440.698023	7542.785906	7564.2186	7799.428116	183698.3704	12177.94397	67850.7644	153102.4226
22	Lidocaine	C14 H22 N2 O	234.17205	Full match	55660012.1	4.631	0.2	1	Full match	3558.324721	7677.213619	21065.7991	70852.00271	66527.50523	62988.11376	38140.37348	46988.67364	42322.85971
23	Lidocaine	C14 H22 N2 O	234.17214	Full match	24953347.9	10.436	0.2	5	Full match	4277.883094	7553.072248	29625.62748	58184.31177	65223.97765	39138.11208	32779.40787	33101.65221	32225.47957
24	Lidocaine	C14 H22 N2 O	234.17212	Full match	14967745.2	9.223	0.2	2	Full match	6162.474638	10157.48288	13959.79457	181038.6064	206005.1047	173366.7787	107831.8217	90814.36708	99583.76164
25	L-N2-[2-Carboxyethyl]arginine	C9 H18 N4 O4	246.134	Invalid mass	2039881.44	8.511	0.2	4	Full match	10074.46323	42197.53068	34536.17784	1838435.064	1840165.563	2197046.852	809348.2358	383226.2184	757982.8363
26	Lupinate	C13 H18 N6 O3	306.14507	Invalid mass	817666.11	8.765	0.2	17	Full match	210899.4481	135307.771	265459.9376	1572266.246	110314.9268	4516098.034	4218000.927	9361298.565	1579619.106
27	Morphine-3-glucuronide	C23 H27 N O9	461.16634	Full match	113518.029	2.716	0.136	0	Full match	8145.650964	14968.13716	870216910.4	30993.44505	3468180.654	3889441.604	29057.67884	74533.01592	209314.7621
28	N-(6-Aminohexanoyl)-6-aminohexanoate	C12 H24 N2 O3	244.17752	Invalid mass	365464.264	8.756	0.2	14	Full match	17474.33384	35435.20549	419829.7547	86127.7336	83654.66737	153054.5289	73105.07214	55009.82317	85237.13987
29	N-(6-Aminohexanoyl)-6-aminohexanoate	C12 H24 N2 O3	244.17747	Invalid mass	375063.776	7.261	0.2	5	Full match	14684.922	33368.37769	7245556.976	1383274099	1499473786	1216924702	1247270529	1334825142	1120498478
30	N,N-Dimethylaniline	C8 H11 N	121.08888	Full match	12382305.9	11.345	0.128	4	Full match	9364.227929	15733.51307	23227.44401	45019.23712	45723.36611	64868.84415	52067.12177	108464.8867	109150.4612
31	N-acetyl-DL-tryptophan	C13 H14 N2 O3	246.10135	Invalid mass	468147.26	2.095	0.2	2	Full match	3339.308537	6034.364743	5317.202821	345639.6657	364637.5939	278384.5016	37843.61988	37804.58455	40030.68247
32	N-Feruloylglycine	C12 H13 N O5	251.07815	Invalid mass	998293.079	9.537	0.2	8	Full match	17474.33384	35435.20549	419829.7547	86127.7336	83654.66737	153054.5289	73105.07214	55009.82317	85237.13987
33	N-Phenylacetylglutamine	C13 H16 N2 O4	264.10989	Full match	211665.814	0.785	0.2	2	Full match	14490.23904	25108.02482	47932.69573	164510.5223	251815.5263	226675.3539	112467.5193	147973.3111	313249.1995
34	Phloretin	C15 H14 O5	274.08499	Full match	5169001.76	9.155	0.2	1	Full match	5691.527795	15105.75003	142711.8238	7177106.382	7682829.65	7728096.192	8008258.983	1232521.089	8289451.378
35	Propionylcarnitine	C10 H19 N O4	217.13034	Full match	324425322	22.574	0.127	21	Full match	19789.4433	36703.11656	892021.071	37558618.96	61571800.25	23833347.41	66153974.83	69900021.42	73736860.97
36	Propionylcarnitine	C10 H19 N O4	217.13035	Full match	3018352.32	14.227	0.2	109	Full match	5802.333421	9887.615814	9294.418214	83359.148	91616.99058	95124.74091	50994.9162	50974.87962	52450.29658
37	Propionylcarnitine	C10 H19 N O4	217.13042	Full match	9214248.55	23.802	0.041	104	Full match	51.87139472	562.9675131	4935.922718	19232.60021	20838.10307	27529.60357	20922.95391	12436.17447	12309.57842
38	Putrescine	C4 H12 N2	88.09972	Full match	983649.57	8.613	0.2	0	Full match	4405.381947	7495.159593	14138.0967	21648.31525	22316.41	22937.97483	18558.26118	20056.29726	19228.71917
39	Pyridoxamine-5'-phosphate;Vitamin B6	C8 H13 N2 O5 P	248.05519	Invalid mass	1567290.33	8.583	0.2	1	Full match	47.94810804	438.3062174	4961.728564	170760.8396	129659.0536	130681.1418	133464.4337	127020.9597	71415.31536
40	S-(Indolylmethylthiohydroximoyl)-L-cysteine	C13 H15 N3 O3 S	293.0828	Invalid mass	884991.343	2.003	0.2	7	Full match	8514.352458	7335.767001	9457.288675	10160.40613	7578.531103	122825.4903	57163.40139	352133.4842	75619.11965
41	S-(Indolylmethylthiohydroximoyl)-L-cysteine	C13 H15 N3 O3 S	293.08281	Invalid mass	6059495.08	1.714	0.2	9	Full match	2605511.516	1089727.611	640317.8728	4287887.624	1413462.486	1129682.269	7630603.914	2051036.706	323208.416
42	S-(Indolylmethylthiohydroximoyl)-L-cysteine	C13 H15 N3 O3 S	293.0843	Invalid mass	3797416.99	1.672	0.2	4	Full match	7753.39364	7561.642388	8451.508096	7607.277502	8074.465558	11638.81103	15112.78642	11291.8442	15938.96101
43	S-Lactoylglutathione	C13 H21 N3 O8 S	379.10494	Full match	183878.788	7.605	0.2	0	Full match	7734.697296	13977.14052	12295.51162	136395.5097	149474.1209	132985.8851	102321.2496	103266.7528	92721.35475

Figure 12. Showing excel sheet composed of metabolite name, formula, molecular weight, area(max), retention time, sample wise area intensity, etc

Potential solution

Check the system for loose fittings. Check pump for leaks, salt buildup, unusual noises. Change pump seals if necessary. Also check make-up of mobile phase and be sure mobile phase is degassed. Purge air from pump head or check valves. Higher column temperatures increase column efficiency. For optimum results, heat eluent before introducing it into the column. Inject smaller volume (e.g., 10 μL vs. 100 μL) or inject the same volume after 1:10 or 1:100 dilutions of sample

Problem 5

If the Resolution is less (step 21).

Potential solution

Prepare a fresh mobile phase and check problem 3 for an obstructed guard or analytical column.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Jaswinder Singh Maras (jassi2param@gmail.com).

Materials availability

The study did not generate any materials.

Data and code availability

The processed data is provided as supplemental information, and the raw data for the manuscript is available on request to the lead contact Dr. Jaswinder Singh Maras (jassi2param@gmail.com).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.101051.

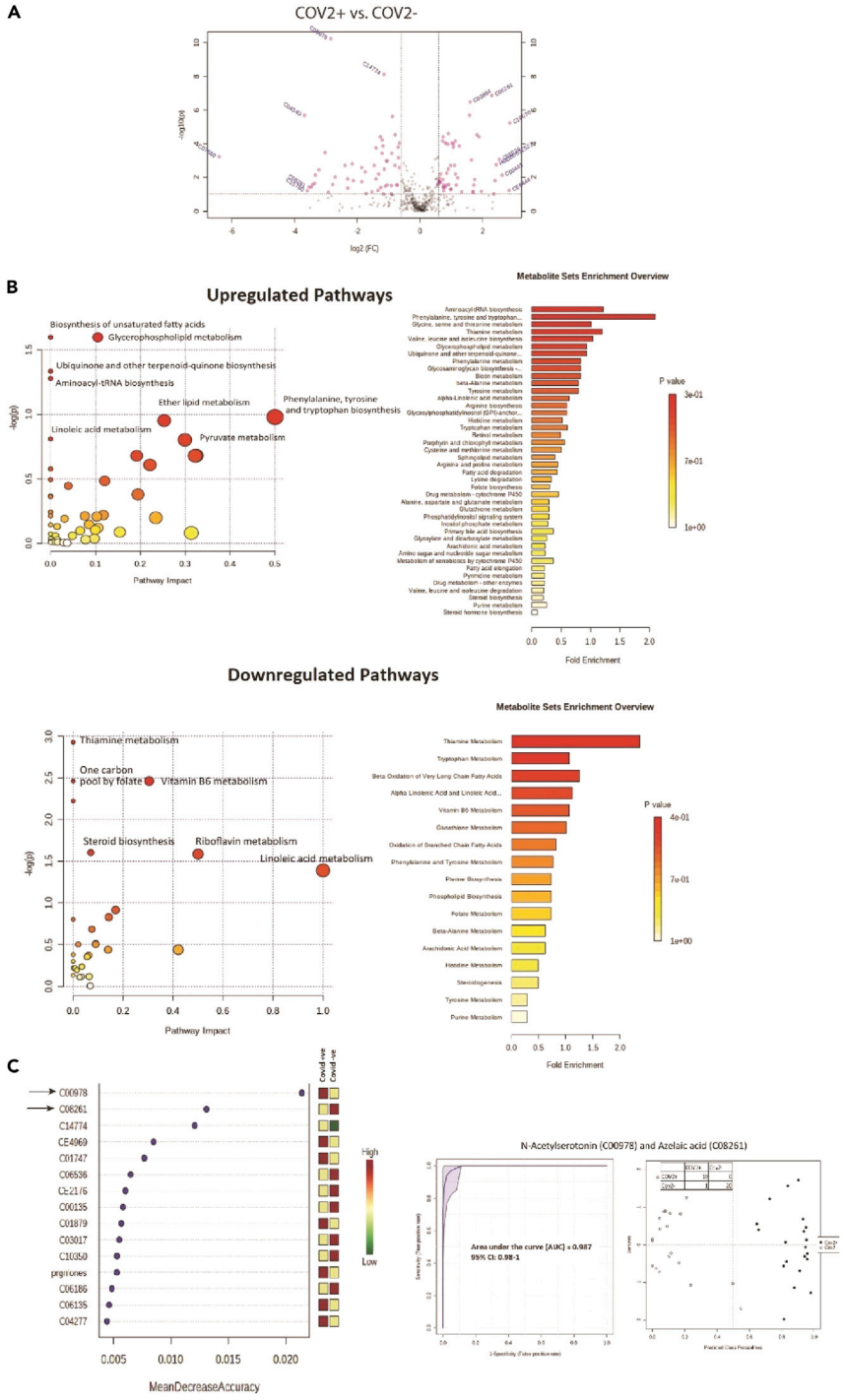


Figure 13. Showing expected outcomes: of VTM metabolome

(A) Volcano plot differentially regulated metabolites in COVID-19 +ve compared to COVID-19 −ve samples ($p < 0.05$, $FC > 1.5$).

(B) Pathway and metabolite set enrichment analysis (KEGG) for the upregulated and downregulated metabolites ($FC > 1.5$, $p < 0.05$) in COVID19-positive respiratory specimen.

(C) Mean decrease in accuracy of the metabolites (Red = upregulated and Green = downregulated and yellow = unchanged) in COVID-19 +ve as compared to COVID-19 −ve. Also, AUROC analysis of N-acetylserotonin (C00978) and azelaic acid (C08261) with AUC = 0.987 CI (0.98–1) $p < 0.05$ along with prediction class probability score plot showing segregation of COVID-19 positive and negative.

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AUTHOR CONTRIBUTIONS

J.S.M. conceptualized the work. The manuscript was written by N.S. and J.S.M., with help from S.H.B., M.Y., G.T., B.M., V.B., S.S., E.G., and S.K.S. The manuscript was read and approved by all authors.

DECLARATION OF INTERESTS

There is no conflict of interest from any of the authors included in the manuscript.

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