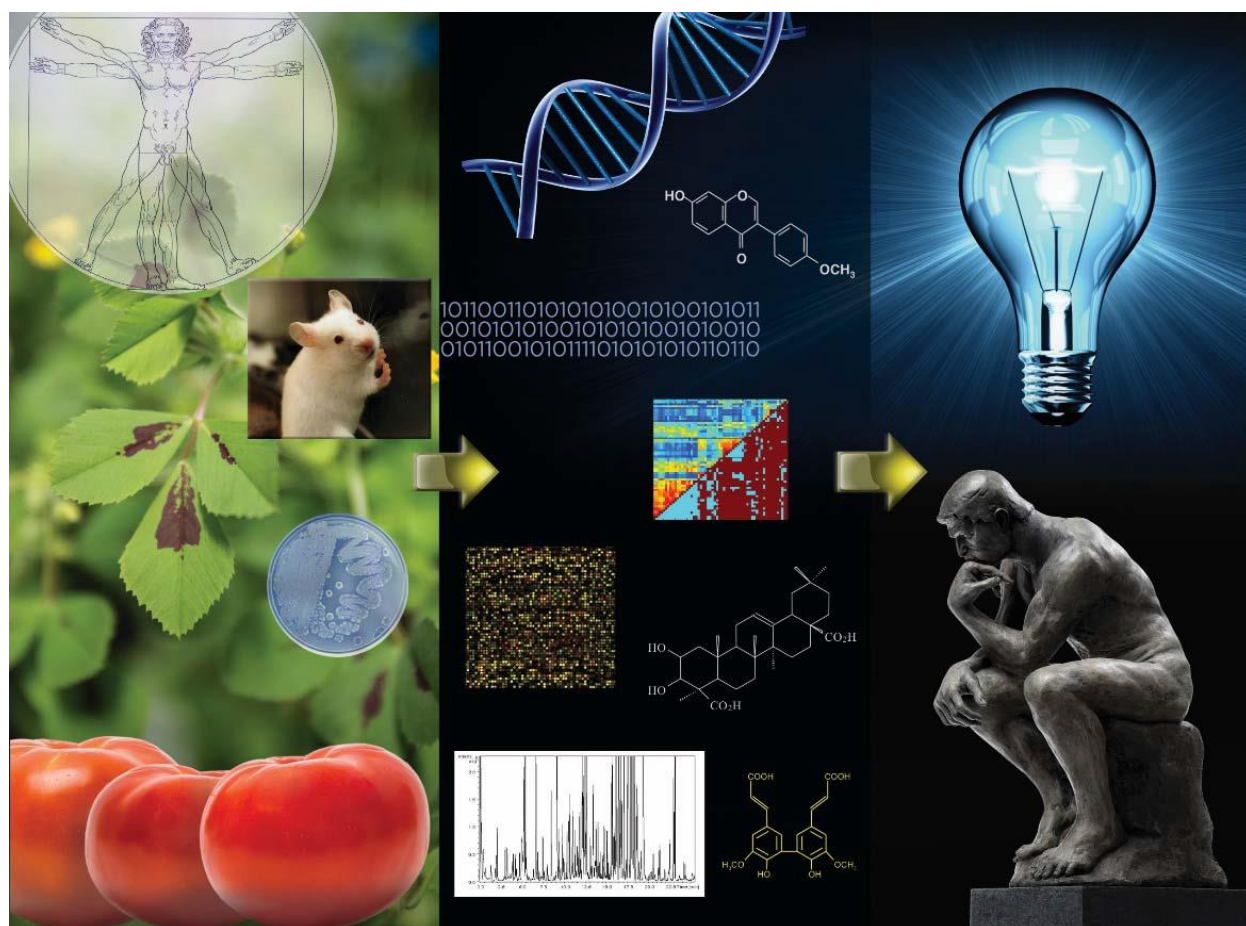


University of Missouri Metabolomics Center

User's Guide



January 2017

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1. The University of Missouri Metabolomics Center (MUMC) and its Staff

The mission of the University of Missouri Metabolomics Center (MUMC) is to provide advanced and cutting edge metabolomics capabilities and expertise to the entire MU community as well as to outside entities. We strive to provide advanced instrumentation and quality service, and to promote collaborations with researchers in plant, animal and medical sciences that seek academic inputs from the metabolomics research group.

MUMC is located in room 243, Christopher S. Bond Life Sciences Center, 1201 Rollins Street, Columbia, MO 65211. Its staff and contact information are listed below.

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2. The University of Missouri Metabolomics Center (MUMC) Instrumentation

MUMC has multiple platforms for targeted and non-targeted small molecule/metabolite analyses. These include:

- Agilent 6890N Gas chromatography (GC) coupled to a 5973N mass selective detector
- Agilent 7890 GC interfaced with 7200B quadrupole time of flight mass spectrometer (GC-QToF MS)
- Bruker Impact II Q-ToF MS coupled to Waters Acquity ultrahigh performance liquid chromatography (UPLC).
- Waters Xevo TQMS (triple quadrupole MS)

3. Current Services Available through the University of Missouri Metabolomics Center (MUMC)

MUMC routine service includes data acquisition and conversion of raw data into NetCDF format. Sample preparation and data processing are not included into the routine service, but can be provided upon user's request at additional cost.

- GC-MS based primary metabolite profiling
- Lignin content and composition analysis

- Cuticle wax analysis
- Plant volatile analysis
- Oil analysis
- LC-MS based secondary metabolite profiling
- Tandem mass spectrometry
- Accurate mass determination
- Plant hormone analysis

MUMC personnel also provide standard and optional data processing. Data processing includes three levels, Tier1, Tier2 and Tier3.

- Tier 1 includes conversion of proprietary instrument raw data into a standardized net.cdf format.
- Tier 2 includes Tier1 plus deconvolution, peak detection, alignment, integration and export of output results into a csv format.
- Tier 3 includes Tier2 plus normalization, outlier detection, PCA, ANOVA, OPLS-DA, *t*-test, fold change, figures and tentative metabolite annotation.

Tier1 is included in the Metabolomics Center routine service. Upon request, the center staff may perform Tier 2 or Tier 3 data processing at an additional cost in addition to the instrumental analysis charges (*ie*, routine service). AMDIS or XCMS is used in data processing, depending on the instrument platform.

4. Sample Submission

- All analyses will require PI approval before analysis.
- An electronic sample submission form or table with sample names, analysis requested and MO code should be provided. This is highly valuable in documenting, tracking, and organizing our output data. All health hazards associated with submitted samples must be fully disclosed.
- Users are highly encouraged to visit with the Center staff prior to sample submission about individual needs. This helps avoid time delays. It also maximizes the quality and probability of success of the data analyses.
- Samples will be analyzed in the order they are received.

5. Sample Storage

A dedicated freezer (-20°C) is located in room 242 to host incoming and completed samples submitted for analysis. Samples should be placed in the freezer together with a sample submission form with the submitter's name and date clearly labeled. After the analysis is complete samples will be returned to the freezer and the submitter will be notified by email that the results are ready and that the samples should be picked up at this time. The samples will be stored for up to 4 weeks after the submitter is notified of the completion of the analysis.

6. Data Storage

Both original raw data and the netCDF format data will be provided to users. It is highly recommended that the users archive their data using CD, DVD or other storage media. The Center will store the data for six months, after which they will be deleted.

7. Fee Schedule

Fees will be charged for each sample analyzed. These fees are summarized below and are intended to recover part of the Center's operation cost such as staff's salaries, columns and solvents.

Type of Analysis	University of Missouri	External Academic and Non-Profit	External Non-Academic and For-Profit
GC/MS Metabolite Profiling Rate	\$46.73	\$63.75	\$109.49
LC/MS Metabolite Profiling Rate	\$46.73	\$63.75	\$125.12
Data Dependent LC/MS/MS	\$46.73	\$63.75	\$125.12
Data Independent LC/MS/MS	\$46.73	\$63.75	\$125.12

Please note the above fees are instrumental costs only. Sample preparation and data processing fees are not included. Upon request, the Center staff may perform sample preparation (metabolite extraction and derivatization) and data processing (peak detection, deconvolution, alignment, peak annotation, quantitation, and multivariate analysis) at additional cost. The costs vary depending on the level of the staff providing the service. The rates are listed below.

Service	University of Missouri	External Non-Profit	For-Profit
Labor Rate 1 (Hourly Tech)	\$ 9.76	\$15.62	\$27.33
Labor Rate 2 (MS Level)	\$13.02	\$20.83	\$36.45
Labor Rate 3 (Ph.D Student)	\$15.53	\$24.84	\$43.47
Labor Rate 4 (Ph.D Level)	\$24.41	\$39.05	\$68.34

8. Workshop and Training

MUMC offers a week-long hands-on workshop every year to familiarize users with metabolomics. The workshop includes lectures of mass spectrometry and metabolomics, and hands-on training. The hands-on training consists of two parts: data acquisition (sample extraction, derivatization and instrumental analysis) and data processing (peak detection, deconvolution, alignment, annotation and quantitation). Each participant will be provided 3 pre-dried, ground and weighed plant tissue samples for hands-on practices. If participant prefers to bring his/her own samples, please dry, grind and weigh them (10 ± 0.06 mg) ahead of time. Only 3 samples are allowed for each participant due to time constraints. Those who wish to bring more samples may bring them for sample processing, but the samples will only be analyzed after the workshop. Participants need to bring their laptop computers on Wednesday and Thursday to perform data processing and analysis.

MUMC also provide customized training throughout the year to those who cannot participate in the annual workshop or wish to take the training at a different time.

There is a fee for both the annual workshop and customized training. The fee is charged to recover consumables, Center staff personnel and instrument time.

9. Collaborative Research

Researchers that seeks in-depth academic inputs from the Center faculty and staff beyond the routine service are encouraged to consider collaboration with the Metabolomics Research group.

Collaboration projects are focused more on unknown identification or large-scale profiling efforts.

Example of collaboration includes:

- xC/MS Metabolic profiling, data processing and multivariate analysis
- Large-scale metabolite annotations
- Identification of unknown metabolites using LC-MS-SPE and NMR
- Method development for specialty metabolites

A short collaborative project description of the project goal, questions asked, answers sought, and time frames of the project is highly desired. This will allow us to assess our ability to answer the questions, such as the availability of personnel, instrumental capacities, the feasibility of solving the problem and the potential impact of the solution. Because of the time and effort dedicated to the project, Center personnel involved in the collaborative project shall be entitled to the authorship of publication.

10. Acknowledgement of the Analytical Chemistry Core Facility Staff support

All documents and publications that include data generated by MUMC should acknowledge MUMC and specific staff involved. MUMC acknowledges and respects the ownership and intellectual rights associated with submitted samples, but reserves the right to publish independently or jointly (preferred) any novel or specialized technical methods, procedures, or protocols developed as part of our interactions and to incorporate these into our ongoing program.

11. Supplies and Chemical Consumables

Users are expected to provide their own sample extraction vials, GC/MS and LC/MS auto sampler vials, caps, inserts, derivatization reagents. Common supplies and reagents are listed below:

- 1-dram (3.84 mL) sample vials, Borosilicate glass, screw-thread, Qorpak* 3.8 ml 144/case, 66009-557 VWR
- Agilent Technologies screw caps and septa, green cap, PTFE/Silicone/PTFE Septa 100/pk, HP-5182-0724 VWR
- Agilent conical glass vial insert. Pulled-point, 100 μ l 100/pk, HP-5183-2085 VWR
- Auto sampler vials, screw-thread, large opening, Agilent Technologies clear vials with write-on spot, 100/pk, HP-5183-2068 VWR
- MSTFA + 1%TMCS, Fisher Scientific, TS-48915

These consumables can also be purchased from MUMC. The prices for these consumables are listed below and may be subject to change without notice.

- 1-dram (3.84 mL) Sample vials: \$0.6/each
- GC sample vial and cap: \$0.39/(vial+cap)
- Inserts: \$0.82/each
- MSTFA+1%TMCS: \$16.38/ampule

12. Protocols

GC-MS Protocols for plant samples (6 mg dry)

Metabolite extraction (6.0 mg dry tissue)

1. Freeze tissue in liquid nitrogen.
2. Lyophilize frozen tissue about 2-3 days (or longer if necessary). The dried tissues should be stored at -80 °C.
3. Homogenize tissue (with glass rod or with mortar and pestle)
4. Weigh dried and ground samples accurately, 6.0-6.05 mg, and transfer to 1-dram (~4 ml) glass vial.
5. Add 1.5 mL of chloroform containing 10.0 µg/mL docosanol (non-polar internal standard) using a glass syringe. Cap the vials tightly, vortex for 1 minute and incubate at 50 °C for 45 minutes in an oven with periodical shaking (shake every 10-15 minutes).
6. Remove samples from the oven, allow samples to equilibrate to room temperature.
7. Add 1.5 ml of water containing 25 µg/mL ribitol (polar internal standard). Cap the vials tightly, vortex for 1 minute and incubate at 50 °C for 45 minutes.
8. Remove samples from the oven, allow samples to equilibrate to room temperature.
9. Centrifuge at 3000xg for 30 minutes at 4 °C to separate the solution into two layers.
10. Use a glass and stainless syringe to transfer 1ml of each layer into 2.0 mL auto-sampler vials. Wash syringe in between samples using chloroform (for organic layer) or methanol (for aqueous layer)
11. Dry the aqueous polar layer (upper layer) in a rotary evaporator and the organic (chloroform) layer under nitrogen. Samples are stored at -80 °C until further processing.

Derivatization and analysis polar metabolites (6 mg dry tissue)

12. Prepare oximation reagent (methoxyamine HCl in pyridine, 15 mg/mL). The reagent needs to be prepared fresh each day. It may require some shaking to dissolve methoxyamine in pyridine. Return methoxyamine bottle to the desiccator after use. Note, the reagent is extremely toxic and should be handled in the fume hood or under a snorkel.
13. Use a glass and stainless syringe to add 50 µL of freshly made methoxyamine reagent into the samples from step 11, cap tightly, briefly sonicate until crystallized metabolites are suspended in solution and incubate at 50 °C for 1h (shake briefly every 10-15 minutes).
14. Remove the sample solutions from the oven and allow them to equilibrate to room temperature.
15. Break an ampoule of MSTFA+1% TMCS. Use a glass and stainless syringe to add 50 µL MSTFA + 1% TMCS to the sample solutions and incubate for 1h at 50 °C (shake briefly every 10-15 minutes). Note, the reagent is extremely toxic and should be handled in the fume hood or under a snorkel.
16. Remove the sample solutions from the oven and allow them to cool down to room temperature. 1.0 µL of the solution is injected at 1:20 split ratio onto a HP 6890N GC equipped with a 60M DB-5-MS column coupled to a HP 5973N MS. The injection port and transfer arm is held at 280 °C, Separation is achieved with a temperature program of 80 °C for 2 min, then ramped at 5 °C/min to 315 °C and held for 12 min, a 60 m DB-5MS column (J&W Scientific, 0.25 mm ID, 0.25 mm film thickness) and a constant flow of 1.0 mL/min. The MS source is held at 250 °C and the quadrupole at 150 °C and scanned from m/z 40-650.

Derivatization and analysis of non-polar metabolites (6 mg of tissue)

17. Re-suspend the non-polar layer samples (from step 11) in 0.8 ml of chloroform and hydrolyze by adding 0.5 mL 1.25 M HCl in MeOH. Cap tightly and incubate at 50 °C for 4 hours. Shake occasionally.
18. Evaporate the solvents and HCl under nitrogen.
19. The samples are then re-suspended in 70 µL pyridine, briefly sonicate until crystallized metabolites are re-suspended in pyridine, and incubate at 50 °C until residue is dissolved.
20. Add 30 µL of MSTFA+ 1% TMCS and incubate 1hr at 50 °C.
21. Equilibrate samples to room temperature, transferred to a 200 µL glass insert using glass pipette and analyze using an Agilent 6890 GC coupled to 5973 MSD scanning from m/z 40-650. 1.0 µL of the solution is injected at 1:1 split ratio. The injection port and transfer arm is held at 280 °C, separation was achieved with a temperature program of 80 °C, for 2 min, then ramped at 5 °C/min to 315 °C and held for 12 min, and a constant flow of 1.0 mL/min.

Note.

- a) Samples should be analyzed within 24 hours of derivatization. So plan your sample derivatization accordingly.
- b) Avoid expose samples and reagents to air. Work fast when adding reagents. Cap tightly after adding reagents. Warm or cool samples to room temperature after removing them from freezer or oven.

GC-MS Protocols for plant samples (1 mg dry)

Metabolite extraction (1.0 mg dry tissue)

1. Freeze tissue in liquid nitrogen.
2. Lyophilize frozen tissue about 2-3 days (or longer if necessary). The dried tissues should be stored at -80 °C.
3. Homogenize tissue (with glass rod or with mortar and pestle)
4. Weigh dried and ground samples accurately, 1.0-1.05 mg, and transfer to 1-dram (~4ml) glass vial.
5. Add 0.5mL of chloroform containing 10.0 µg/mL docosanol (non-polar internal standard) using a glass syringe. Cap the vials tightly, vortex for 1 minute and incubate at 50 °C for 45 minutes in an oven with periodical shaking (shake every 10-15 minutes).
6. Remove samples from the oven, allow samples to equilibrate to room temperature.
7. Add 0.5ml of water containing 25 µg/mL ribitol (polar internal standard). Cap the vials tightly, vortex for 1 minute and incubate at 50 °C for 45 minutes.
8. Remove samples from the oven, allow samples to equilibrate to room temperature.
9. Centrifuge at 3000xg for 30 minutes at 4 °C to separate the solution into two layers.
10. Use a glass and stainless syringe to transfer 1ml of each layer into 2.0 mL auto-sampler vials. Wash syringe in between samples using chloroform (for organic layer) or methanol (for aqueous layer)
11. Dry the aqueous polar layer (upper layer) in a rotary evaporator and the organic (chloroform) layer under nitrogen. Samples are stored at -80 °C until further processing.

Derivatization and analysis polar metabolites (1 mg dry tissue)

12. Prepare oximation reagent (methoxyamine HCl in pyridine, 15 mg/mL). The reagent needs to be prepared fresh each day. It may require some shaking to dissolve methoxyamine in pyridine. Return methoxyamine bottle to the desiccator after use. Note, the reagent is extremely toxic and should be handled in the fume hood or under a snorkel.
13. Use a glass and stainless syringe to add 40 µL of freshly made methoxyamine reagent into the samples from step 11, cap tightly, briefly sonicate until crystallized metabolites are suspended in solution and incubate at 50 °C for 1h (shake briefly every 10-15 minutes).
14. Remove the sample solutions from the oven and allow them to equilibrate to room temperature.
15. Break an ampoule of MSTFA+1%TMCS. Use a glass and stainless syringe to add 40 µL MSTFA + 1% TMCS to the sample solutions and incubate for 1h at 50 °C (shake briefly every 10-15 minutes). Note, the reagent is extremely toxic and should be handled in the fume hood or under a snorkel.
16. Remove the sample solutions from the oven and allow them to cool down to room temperature. 1.0 µL of the solution is injected at 15:1 split ratio onto a HP 6890N GC equipped with a 60M DB-5-MS column coupled to a HP 5973N MS. The injection port and transfer arm is held at 280 °C, Separation is achieved with a temperature program of 80 °C for 2 min, then ramped at 5 °C/min to 315 °C and held for 12 min, a 60 m DB-5MS column (J&W Scientific, 0.25 mm ID, 0.25 mm film thickness) and a constant flow of 1.0 mL/min. The MS source is held at 250 °C and the quadrupole at 150 °C and scanned from m/z 50-650.

Derivatization and analysis of non-polar metabolites (1 mg of tissue)

17. Re-suspend the non-polar layer samples (from step 11) in 0.4 ml of chloroform and hydrolyze by adding 0.5 mL 1.25 M HCl in MeOH. Cap tightly and incubate at 50°C for 4 hours. Shake occasionally.
18. Evaporate the solvents and HCl under nitrogen.
19. The samples are then re-suspended in 35 µL pyridine, briefly sonicate until crystallized metabolites are re-suspended in pyridine, and incubate at 50°C until residue is dissolved.
20. Add 30 µL of MSTFA+ 1% TMCS and incubate 1hr at 50°C.
21. Equilibrate samples to room temperature, transferred to a 200 µL glass insert using glass pipette and analyze using an Agilent 6890 GC coupled to 5973 MSD scanning from m/z 40-650. 1.0 µL of the solution is injected at 1:1 split ratio. The injection port and transfer arm is held at 280°C, separation was achieved with a temperature program of 80°C, for 2 min, then ramped at 5°C/min to 315°C and held for 12 min, and a constant flow of 1.0 mL/min.

Note

- c) Samples should be analyzed within 24 hours of derivatization. So plan your sample preparation accordingly. Avoid preparing more than 24 samples at one time.
- d) Avoid expose samples and reagents to air. Work fast when adding reagents. Cap tightly after adding reagents. Warm or cool samples to room temperature after removing them from freezer or oven.

LC-MS Protocols for plant samples (10 mg dry)

1. Freeze tissues in liquid nitrogen.
2. Lyophilize frozen tissue about 2-3 days (or longer if necessary). The dried tissues should be stored at -80 °C until use.
3. Grind the dry tissue using mortar and pestle.
4. Weigh tissue ($10\text{mg} \pm 0.06$) into a 1-dram glass vial (3.8 mL).
5. Add 1.0 mL of 80% methanol containing internal Standard (0.018 mg/mL umbelliferone).
6. Gently shake on orbital shaker for 2 hours.
7. Centrifuge samples for 30 minutes at 3000g at 4 °C.
8. Transfer 500 μl to sample vial.
9. Store sample at -20 °C until sample analysis.
10. Inject 2 μl into LC-MS. Separation is achieved on an ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 μm , 2.1 mm X 150 mm. Column temperature: 60 °C, mobile phase A: 0.1% formic acid, B: acetonitrile. Flow rate: 0.56 mL/min. Metabolites are detected using a Bruker Impact II Q-TOF MS with a scan range from m/z 100 -1500.

Combined GC-MS and LC-MS Protocols for plant samples (10 mg dry)

1. Add 1.0 mL of 80% MeOH containing 18 µg/mL umbelliferone to the samples, sonicate for 5 seconds and agitate samples for 2 hours.
2. Transfer 0.5 mL of supernatant to glass sample vial for UPLC-MS analysis and follow the above LC-MS Protocols for sample analysis. Add 1.5 mL of CHCl₃ with 10 µg/mL docosanol to the remaining sample and continue to incubate at 50 °C for 1 hour.
3. Add 1.0 mL of HPLC Grade Water with 25 µg/mL ribitol and incubate at 50 °C for 1 hour.
4. Centrifuge for 40 minutes at 3000 g at 10 °C.
5. Transfer 1 mL of polar phase (upper layer) to glass sample vial, dry under nitrogen. Transfer 1 mL of nonpolar phase (lower layer) to glass vial, dry under nitrogen. Store dry samples at -20 °C.
Note, wash syringe in between samples.
6. Follow the above protocol to process the polar and non-polar samples.