Issued: 07-24-2011 Valid from: 07-262011 Validation: Fiehn	Validity area: UC Davis Genome Center, Metabolomics Core and Research Laboratories
Responsible: Fiehn	
This SOP supersedes: GC-TOF QC_09202006	approved: Fiehn

Evaluation of quality controls for using GC-TOF Pegasus III for metabolomic purposes

1. Objective:

Routine GC-TOF measurements using the GC-TOF Pegasus III instrument for "metabolomics" and "target analysis".

Establish **in-control quality evaluation** for

- amino acid analysis
- hydroxyl acid analysis
- carbohydrate analysis
- 2-step derivatization performance
- high boiling point compounds
- low boiling point compounds
- overall method sensitivity
- column performance
- absolute quantification for all compounds in the QC mixture by 6-point calibration.

2. References:

Fiehn O, Wohlgemuth G, Scholz M, Kind T, Lee DY, Lu Y, Moon S, Nikolau BJ (2008) Quality control for plant metabolomics: Reporting MSI-compliant studies. Plant J. 53, 691-704

3. Instruments:

- GC-TOF Leco Pegasus III instruments "a (core lab)" and "b (research lab)" , purchased in 2004
- fine balance with an accuracy of ± 0.01 mg
- ultrasonicator
- Centrifuge Eppendorf 5415 D
- calibrated pipettes 0.1-2 µl, 0.5-10 µl, 1-200µl and 100-1000µl
- Eppendorf tubes 2ml, uncolored, cat. # 022363204
- ThermoElectron Neslab RTE 740 cooling bath at –20°C
- MiniVortexer VWR.
- Orbital Mixing Chilling/Heating Plate, Torrey Pines Scientific Instruments
- Speed vacuum concentration system,.Labconco Centrivap cold trap

4. Chemicals and consumables

- Fatty acid methyl esters C08-C30 at 2.5 – 5.0 µg/ml from a reference solution of 0.8 mg/ml C08, C09, C10, C12, C14, C16 and 0.4 ug/ml C18, C20, C22, C24, C26, C28, C30 in chloroform for retention index calculation according to document "LAB standard protocols"
- Quality control mixtures as six-point calibration curves according to document "LAB standard protocols" using 0.1, 0.25, 0.5, 1, 2.5 and 5 ug/ml (QC01-QC06) of a reference solution of compounds according to "SOP qcmix 02032009", namely: Pyruvate, alanine, valine, serine, nicotinic acid, succinic acid, methionine, aspartic acid, 4-hydroxyproline, salicylic acid, glutamic acid, creatinine, alpha

ketoglutaric acid, N-acetyl-l-aspartic acid, asparagine, putrescine, shikimic acid, citric acid, lysine, glucose, stearic acid d35, glucose-6-phosphate, arachidic acid, serotonin, adenosine, sucrose, chlorogenic acid, alpha tocopherol, cholesterol

- Pyridine (Acros Organics; Cat. No. 270970), N-methyl-N-(trimethylsilyl)-trifluoroacetamide [MSTFA], (Aldrich; Cat. No. 394866), Methoxyamine hydrochloride [MeOX] (Aldrich; Cat. No. 226904)
- Use 1 ml bottles of MSTFA to prevent using outdated, partially oxidized or hydrolyzed reagents.

5. **Procedure:**

(A) Maintenance and quality control (**system performance check**)

1. ***Read the log file of the last 24 hours of operation*** to check for hardware error messages or autotune / calibration problems. No injection must be performed before hardware errors are removed. This check needs to include that files have been transferred correctly to the data servers.
2. Check that the instrument's computer hard disk has enough space to store new raw data.
3. Every 10 samples: exchange Gerstel multi-baffled split/splitless liners via Gerstel Maestro software and MPS2.
4. After change, deactivate each liner by injecting reagent blanks three times with rapid GC-flash program.
5. Clean syringe, waste solvent vials and washing solvent vials weekly with ethyl acetate weekly.
6. Clean vacuum pump air filters once a year.
7. Vacuum oil pump maintenance: check oil level and color and viscosity quarterly and change at least once a year.
8. Check gas leaks when cutting or changing a column. (oxygen/helium ratio m/z 32 to m/z 4 < 3, nitrogen/helium ratio m/z 28 to m/z 4 < 5).
9. Cut column if QC evaluation is not in control.
10. Exchange O-rings for liners, liner heads, syringes, column, filters for injector tubings, injector silver plates, filaments and other replacement parts as necessary, e.g., when QC evaluation is not in control.

(B) Acquire quality control data

Concentrate a six-point dilution series of the quality control mixture to complete dryness in standard Eppendorf snap cap tubes. When using previously prepared QC samples out of the freezers, make sure that QCs have reached room temperature before opening them (otherwise, water will condense inside the tubes and render MSTFA unsuitable.) Derivatize each QC with 10 ul of 30 mg/ml methoxyamine in pyridine for 60 min at 30°C, and subsequently with 90 ul of MSTFA for 30 min at 37°C according to SOP "derivatization 10212010". The pyridine and the MSTFA must not be older than 2 months. Add 1 ul of C08-C30 FAME retention index marker mixture. Transfer mixtures to standard GC-autosampler vials with crimp caps. Use one reagent blank and one method blank control before acquiring QC series data, and and one method blank after the QCs. Evaluate the QCs right after the run by visual inspections. No injection of samples must be performed if evaluation of chromatograms reveals that the instrument is not in control.

Acquire GC-TOF MS chromatograms for the quality control standard mixture ("QC mix") using splitless injection of 0.5 ul with 25 seconds splitless time into multibaffled liners at 40°C using a 30 meter 0.25 mm i.d. rtx-5SilMS column (Restek) with 0.25 um film thickness and 10 m integrated, empty guard colum. Use 1.0 ml Helium constant flow. Use GC temperature ramp t 0-1 min=50°C, ramp 20°C/min to 330°C, hold 330°C for 5 min. Adjust the default MS delay of 330 s according to the actual end of the pyridine/MSTFA solvent peak. Acquire data from m/z 85-500 at 17 spectra/s at a default of 1850 V detector voltage at 70 eV filament emission and 250°C source temperature with 280°C transfer line temperature.

Use a six-point calibration curve for each new batch of analyses starting from the lowest concentration (QC01) to the highest (QC06). Name the files ***yymmddaopqc01*** **to** ***yymmddaopqc06*** for the core machine, resp. ***yymmddbopqc01*** **to** ***yymmddbopqc06*** for the research machine with yy=year, mm=month, dd=day, op=operator according to your name definition in miniX, qc=quality control.

If your samples or your QCs are deviating from SOPs and are not meant to be used for QC monitoring (for

example, when testing new liners or new derivatization protocols), use different file names. If you wish these samples to be processed through miniX/BinBase, use sa = sample as acronyms, instead of qc=quality controls.

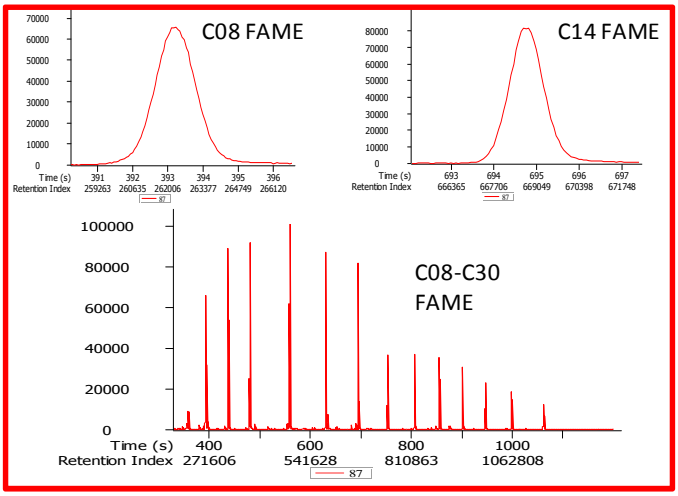
(C) Assess absolute and relative machine sensitivity and performance

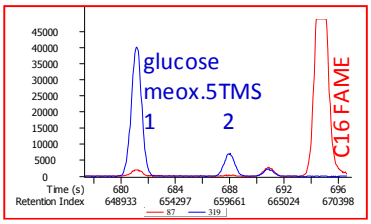
(1) Plot the series of internal retention index markers **C08-C30 FAMEs** at m/z 87.

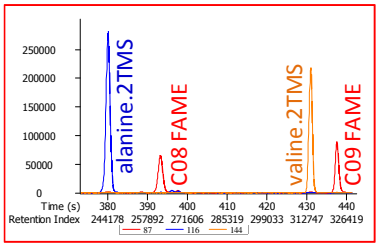
- The peak intensity distributions should follow the graph below.
- The intensity of C14 fatty acid methyl ester at m/z 87 should be >80,000 counts per spectrum (cps) under the conditions described above. The intensity of C30 FAME at m/z 87 should be >10,000 counts.
- Peak shapes of all FAMEs should be strictly Gaussian-type without peak tailing at the base with peak widths at ca. 3s. For evaluation of peak shapes, zoom out C08 and C14 FAME. Peak tailing of C14 FAME may indicate column aging. Peak distortions of C08 FAME may indicate column aging as well as problems during injections, e.g., pressure pulses, valve closure times or other injector malfunctions.

(2) Plot **glucose 1 and glucose 2** peaks at m/z 319. Two peaks should be visible roughly 6s and 13s before C16 FAME. This test validates that the methoximation reaction has worked properly

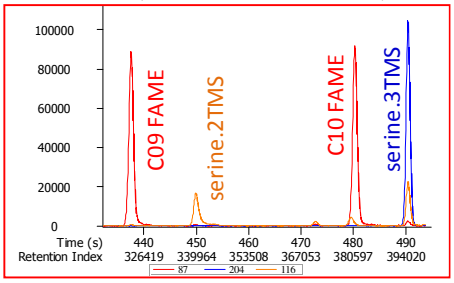
(3) Plot **alanine.2TMS** at m/z 116 and **valine.2TMS** at m/z 144. Peaks should be Gaussian-shaped and alanine should be about the same height as valine. This test validates that there is no injection discrimination or peak distortions for low boiling point compounds.

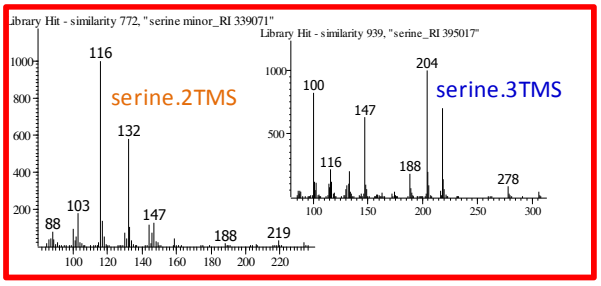




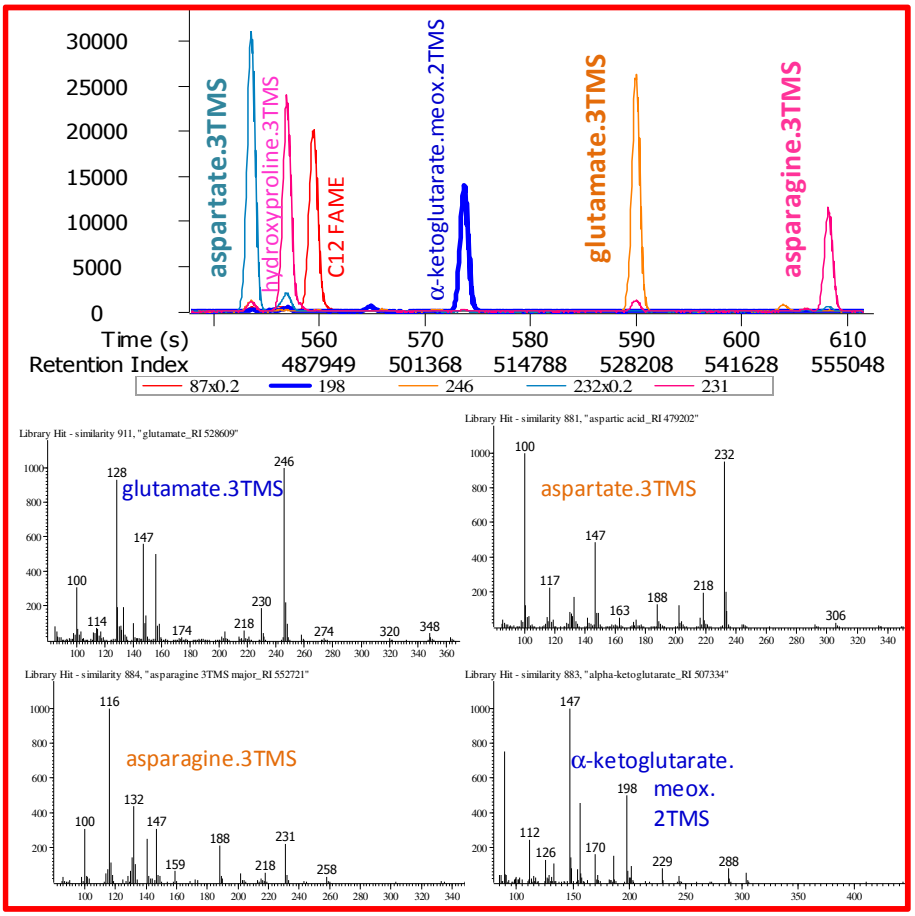


(4) Plot **serine.3TMS** at m/z 204 and **serine.2TMS** at m/z 116. The ratio should be about 3:1 or higher, but at least 2:1 (lower intervention limit).





(5) Plot **aspartate**.3TMS at m/z 232 (minimize x0.2), **asparagine**.3TMS at m/z 231, **glutamate**.3TMS at m/z 246, **alpha-ketoglutarate**.meox.2TMS at m/z 198 and **oxoproline**.2TMS at m/z 156 for a freshly prepared QC06 sample.



Attention: glutamate converts to oxoproline while standing in the autosampler, as well as catalyzed by contaminations in the liner or other parts that are involved in the injection.
A range of other metabolites elute in this retention window, e.g. N-acetylaspartate, creatinine, oxoproline, methionine, salicylate (all not shown here), as well as hydroxyproline (shown).

Validation criteria:
aspartate/α-ketoglutarate should be > 8 : 1 (lower intervention limit)
glutamate/α-ketoglutarate should be > 1 : 1 (lower intervention limit)
asparagine/α-ketoglutarate should be > 0.8 : 1 (lower intervention limit)

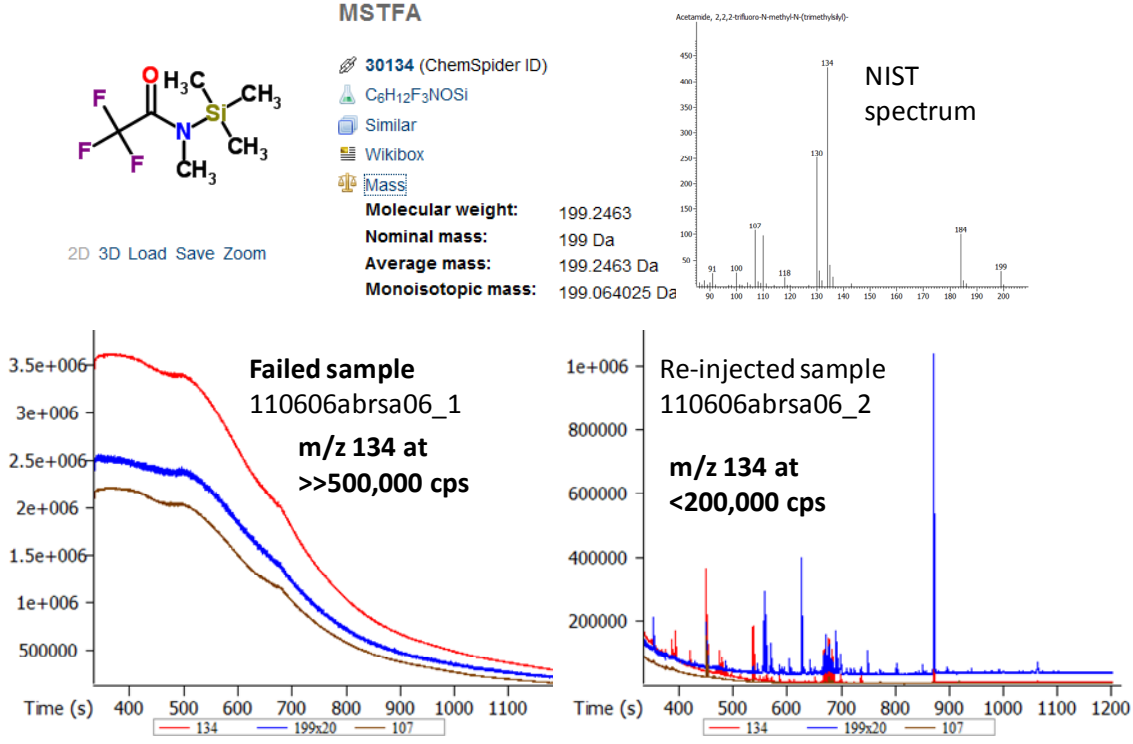
This test validates there is no injection discrimination against amino acids. If test fails, cut the empty guard column by 10 cm and repeat test. Any accumulated dirt in the guard column discriminates against amino acid derivatization status. Aspartate must never fail the test. In very severe cases, putrescine (m/z 174, not shown here) to α-ketoglutarate ratios might fall below < 20:1. No sample injection can be performed in such cases.
Asparagine might fail the validation criteria first, followed by glutamate and serine. Consult Prof. Fiehn and

laboratory manager Mine Palazoglu for counter measures. Problems are more readily apparent at low concentration QCs (QC01-03) compared to QC06.
A full evaluation report will be available by BinBase.

8. Failures and errors
(1) Check if the **log file** reads 'hardware error'. No injection can be done if manual inspection of mass calibration confirms major errors that were reported in the log file.

(2) Check all chromatograms for **gross errors** in ***'no baseline subtraction'***.
Example below: gross breakthrough of MSTFA, inhibiting correct transfer of sample onto the column.

Validation criteria: mass trace m/z 134 must be < 200,000 cps after MS delay time. The lower, the better (average over injection series should be <100,000 with a usual readout of <50,000).



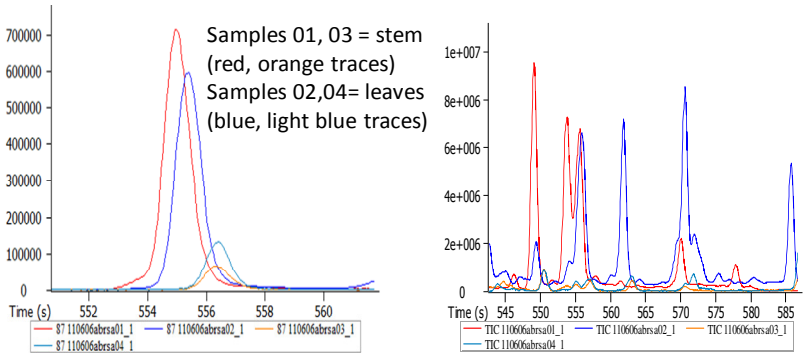
Attention: sample peaks may be present if m/z 134 is >1.5e06, however, sample is still incorrectly transferred onto the column! Therefore, ***look at all samples, visually, the day after data acquistion*.**
Many problems are directly apparent even in 'background subtraction' view, see below.

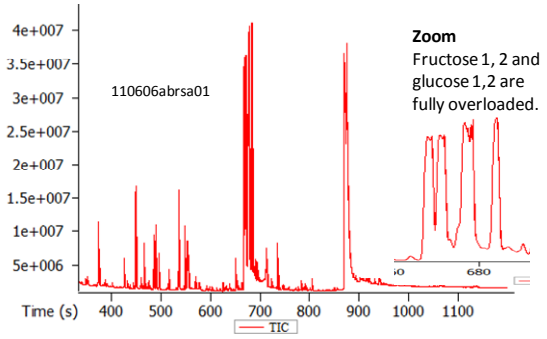
- The intensity of FAMEs and the retention time of FAMEs must be closely matching. This was not the case for comparing samples 01,03 or samples 02,04 (see example).

- Similar samples (e.g. 'plant leaves/plant leaves' or 'plant stems/plant stems') should roughly look alike. This was not the case for comparing samples 01,03 or samples 02,04 (see example).

(3) Sample overload.
Before running large sequences of samples, ensure that samples are not overloaded onto column. Plant samples are often overloaded by sugars, i.e. glucose, fructose, sucrose, but other samples (even cell pellets, plasma, tissue

extracts) may also be overloaded, especially when users were not informed about optimal amounts to be used.





Several problems are caused by sample overloading:
(a) overloaded peaks cannot be quantified.
(b) overloaded peaks may cause RI drifts in adjacent compounds, causing huge problems in BinBase data processing.
(c) Adjacent peaks suffer deconvolution problems and may not be found in BinBase processing.
(d) Overloaded peaks may have very skewed spectra and may not be found in BinBase processing or even cause the generation of new Bins.

Collaborators and lab members may not easily see such problems in data sheets. Nevertheless, it must be strictly avoided that collaborators or lab members approach the Metabolomics Facility with QC / QA problems that the instrument operators were not aware of! Especially extramural clients may send duplicate samples in blinded fashion, which can rightfully be expected to yield very similar data.

Strict rule of work:
For every new study, a single representative sample is extracted, the extract is aliquoted into 2 aliquots, but only 1 aliquot is derivatized and analyzed. Rule of thumb: Peaks that have TIC > 1e7 may be overloaded. If chromatograms are overloaded like here, less extract is used (e.g. 1/5), still using splitless injection.
Attention: do not use split injections. Split injections (e.g. 1/5) will cause amino acids to not comply to QC criteria.
Subsequently, Prof. Fiehn is consulted, by email copy (pptx) in case he is not in office. In a few cases (e.g. urea in urine without urease treatment, or sucrose in rice leaf extracts), a single compound may be intentionally left overloaded in order to detect lower abundant metabolites.

9. Quality assurance measures
Report instrument maintenance measures and any irregularities in the 'instrument maintenance book'.

10. Waste disposal
Chemicals are disposed into appropriate bottles under the fume hood before monthly disposal collection. Glass vials and consumables are collected and stored under the fume hood before monthly disposal. Other GC-TOF waste (rubber seals, O-rings etc) can be disposed into regular waste.