

Supplementary material:

Modified protocol of harvesting, extraction and normalization approaches for gas chromatography mass spectrometry-based metabolomics analysis of adherent cells grown under high fetal calf serum conditions

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Table S1. Individual metabolite relative standard deviation (RSD) of HCT116 cells cultured in 10% and 20% FCS conditions using different quenching solvents, extraction ratios and polar volumes. *: phosphate.

| Condition name | 50_LOW | | 50_MEDIUM | | 50_HIGH | | 80_HIGH | |
|-------------------------|--------|-----|-----------|----|---------|----|---------|----|
| FCS [%] | 10 | 20 | 10 | 20 | 10 | 20 | 10 | 20 |
| Biological replicates | 4 | 5 | 3 | 3 | 4 | 4 | 5 | 4 |
| Alanine | 83 | 38 | 13 | 35 | 32 | 27 | 28 | 55 |
| Asparagine | 69 | 111 | 8 | 48 | 27 | 48 | 19 | 43 |
| Aspartic acid | 95 | 32 | 2 | 39 | 20 | 29 | 14 | 49 |
| Glutamine | 69 | 49 | 32 | 51 | 6 | 25 | 23 | 16 |
| Glycine | 70 | 31 | 22 | 55 | 8 | 21 | 17 | 40 |
| Isoleucine | 63 | 36 | 35 | 70 | 24 | 24 | 20 | 16 |
| Leucine | 70 | 30 | 18 | 53 | 7 | 19 | 13 | 27 |
| Lysine | 72 | 131 | 48 | 87 | 50 | 29 | 55 | 58 |
| Methionine | 66 | 66 | 48 | 93 | 41 | 27 | 48 | 15 |
| Phenylalanine | 67 | 30 | 20 | 58 | 6 | 23 | 13 | 25 |
| Proline | 67 | 29 | 21 | 70 | 7 | 21 | 31 | 57 |
| Serine | 68 | 47 | 24 | 67 | 20 | 20 | 21 | 21 |
| Threonine | 105 | 37 | 9 | 30 | 16 | 29 | 29 | 63 |
| Tryptophan | 42 | 70 | 34 | 63 | 52 | 22 | 32 | 38 |
| Tyrosine | 68 | 62 | 19 | 54 | 18 | 66 | 42 | 61 |
| Valine | 67 | 31 | 20 | 62 | 8 | 19 | 13 | 63 |
| Fructose-6-phosphate | | 39 | 40 | 70 | | 67 | 25 | 86 |
| Glucose-6-phosphate | 73 | 56 | 24 | 61 | 21 | 35 | 30 | 59 |
| Glyceric acid-3-P* | 90 | 32 | 16 | 59 | 34 | 74 | 112 | 23 |
| Phosphoenolpyruvic acid | 117 | 27 | 10 | 50 | 11 | 31 | 129 | 43 |
| Pyruvic acid | 68 | 58 | 27 | 63 | 25 | 57 | 30 | 25 |
| Dihydroxyacetone P* | | 38 | | 95 | | | | |
| Glycerol | 66 | 32 | 18 | 49 | 4 | 18 | 15 | 12 |
| Glycerol-3-phosphate | 64 | 29 | 32 | 54 | 10 | 20 | 25 | 24 |
| Adenine | 73 | 32 | 21 | 54 | 8 | 26 | 28 | 12 |
| Adenosine | 155 | 103 | 131 | 63 | 35 | 77 | 103 | 15 |
| Uracil | 65 | 36 | 29 | 66 | 16 | 26 | 22 | 26 |

| | | | | | | | | |
|---------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| Butanoic acid, 3-hydroxy- | 73 | 35 | 26 | 63 | 40 | 23 | 18 | 3 |
| Butanoic acid, 4-amino- | 63 | 31 | 16 | 57 | 7 | 27 | 20 | 51 |
| Erythritol | 66 | 32 | 20 | 54 | 9 | 21 | 12 | 24 |
| Glutaric acid | 64 | 29 | 34 | 54 | 12 | 19 | 11 | 29 |
| Glyceric acid | 152 | 31 | 23 | 133 | 11 | 50 | 12 | 50 |
| Ribose | 75 | 44 | 26 | 33 | 12 | 22 | 25 | 22 |
| Ribose-5-phosphate | | 33 | | 37 | | 78 | | 47 |
| Citric acid | 67 | 31 | 20 | 49 | 105 | 21 | 83 | 181 |
| Fumaric acid | 63 | 32 | 24 | 57 | 12 | 27 | 22 | 28 |
| Glutaric acid, 2-hydroxy- | 91 | 30 | 25 | 48 | 25 | 48 | 54 | 44 |
| Glutaric acid, 2-oxo | 66 | 34 | 28 | 50 | 10 | 26 | 23 | 28 |
| Malic acid | 68 | 32 | 22 | 55 | 8 | 20 | 14 | 30 |
| Succinic acid | 66 | 32 | 21 | 54 | 9 | 21 | 14 | 29 |
| Glutamic acid | 72 | 33 | 15 | 66 | 15 | 27 | 46 | 98 |
| Median RSD | 68 | 33 | 22 | 55 | 14 | 26 | 23 | 30 |

Table S2. Individual metabolite relative standard deviation (RSD) of HUVECs cultured in 20% FCS conditions using different quenching solvents, extraction ratios and polar volumes for the different protocols. Cells were measured in different batches (dashed line). *: 50_Medium.

| Condition name | 50_LOW | 50_MED* | 50_MED* | 80_HIGH | 50_HIGH | 80_HIGH |
|------------------------------|---------------|----------------|----------------|----------------|----------------|----------------|
| Biological replicates | 2 | 3 | 3 | 3 | 2 | 3 |
| Alanine | 41 | 22 | 39 | 16 | 27 | 26 |
| Asparagine | | | | | 8 | 84 |
| Aspartic acid | 51 | 75 | 41 | 33 | 39 | 55 |
| Glutamine | | | | | 19 | 85 |
| Glycine | 13 | 18 | 42 | 21 | 4 | 24 |
| Isoleucine | | 40 | 44 | 59 | 55 | 17 |
| Leucine | | 49 | 43 | 59 | 7 | 79 |
| Lysine | 51 | 50 | 34 | 10 | 9 | 111 |
| Methionine | 18 | | 31 | 51 | 42 | 73 |
| Phenylalanine | 9 | 21 | 35 | 28 | 7 | 56 |
| Proline | 121 | 73 | 47 | 13 | 32 | |
| Serine | 50 | 34 | 41 | 39 | 8 | 61 |
| Threonine | 8 | 67 | 33 | 31 | 14 | 14 |
| Tyrosine | | | | | | 147 |
| Valine | 17 | | 44 | 17 | 6 | 71 |
| Glyceric acid-3-phosphate | | | | | 23 | 54 |
| Lactic acid | 10 | 11 | 31 | 17 | 7 | 8 |
| Phosphoenolpyruvic acid | 98 | | 77 | 16 | 6 | 37 |
| Pyruvic acid | 9 | 2 | 48 | 32 | 6 | 9 |
| Glycerol | 4 | 11 | 36 | 13 | 4 | 5 |
| Glycerol-3-phosphate | 26 | 50 | 45 | 13 | 35 | 46 |
| Adenine | 11 | 27 | | 44 | 1 | 35 |
| Adenosine | | 26 | 35 | 17 | | |
| Cytosine | 29 | | 50 | 5 | | |

| | | | | | | |
|---------------------------|-----------|-----------|-----------|-----------|-----------|-----------|
| Uracil | | | 31 | 19 | | 120 |
| Butanoic acid, 3-hydroxy- | 19 | 13 | 38 | 8 | 20 | 16 |
| Butanoic acid, 4-amino- | | | | | 49 | 137 |
| Erythritol | 10 | 15 | 39 | 12 | 12 | 9 |
| Glutaric acid | 26 | | 15 | 12 | 21 | 15 |
| Glyceric acid | 22 | 16 | 31 | 8 | 43 | 36 |
| Ribose | 13 | 22 | 39 | 34 | 10 | 21 |
| Ribose-5-phosphate | | | 29 | | | 69 |
| Citric acid | 36 | 63 | 38 | 15 | 11 | 36 |
| Fumaric acid | | 51 | 38 | 4 | 85 | 7 |
| Glutamic acid | 29 | | 96 | 59 | 21 | 74 |
| Glutaric acid, 2-hydroxy- | 1 | 37 | 67 | 18 | 19 | 26 |
| Glutaric acid, 2-oxo- | 7 | 5 | | 33 | 5 | 15 |
| Malic acid | 32 | 25 | 39 | 5 | 1 | 14 |
| Succinic acid | 16 | 15 | 32 | 11 | 31 | 10 |
| Median RSD | 18 | 26 | 39 | 17 | 13 | 36 |

Table S3. Median of the relative standard deviation (RSD) per metabolite of HUVECs cultured in 20% FCS conditions using different quenching solvents, extraction ratios and polar volumes for the different protocols. Cells were measured in different batches.

| Condition name | 50_LOW | 50_MEDIUM | 50_HIGH | 80_HIGH |
|------------------------------|----------|-----------|----------|----------|
| Biological replicates | 2 | 3 | 2 | 3 |
| Male HUVEC | 18% | 26% | | |
| Female HUVEC | | 39% | | 17% |
| Female HUVEC | | | 13% | 36% |

Table S4. Percentage of metabolites with a relative standard deviation (RSD) < 30% of HUVECs cultured in 20% FCS conditions using different quenching solvents, extraction ratios and polar volumes for the different protocols. Cells were measured in different batches.

| Condition name | 50_LOW | 50_MEDIUM | 50_HIGH | 80_HIGH |
|------------------------------|----------|-----------|----------|----------|
| Biological replicates | 2 | 3 | 2 | 3 |
| Male HUVEC | 68% | 54% | | |
| Female HUVEC | | 6% | | 67% |
| Female HUVEC | | | 74% | 44% |

Table S5. Relative standard deviation (RSD) of measured protein amount of HUVECs cultured in 20% FCS conditions using different quenching solvents, extraction ratios and polar volumes for the different protocols. Cells were measured in different batches.

| Condition name | 50_LOW | 50_MEDIUM | 50_HIGH | 80_HIGH |
|------------------------------|----------|-----------|----------|----------|
| Biological replicates | 2 | 3 | 2 | 3 |
| Male HUVEC | 49% | 15% | | |
| Female HUVEC | | 38% | | 9% |
| Female HUVEC | | | 19% | 9% |

Table S6. Median of the relative standard deviation (RSD) per metabolite and percentage of metabolites with a RSD < 30% of HUVECs (three biological replicates) cultured in 20% FCS conditions.

| Normalization Strategy | Cell mass | Protein |
|------------------------|-----------|---------|
| Median RSD [%] | 61% | 52% |
| RSD < 30% [%] | 18% | 26% |

Table S7. List of metabolite derivatives and their biological group used for reference search. AA: Amino acids. PPP: Pentose phosphate pathway. TCA: Tricarboxylic acid cycle. TMS: Trimethylsilyl derivatives. MeOX: Methoxyamine hydrochloride.

| Group | Metabolite | Detected as |
|------------|----------------------------|-------------|
| AA | Alanine | 3TMS |
| | | 2TMS |
| AA | Asparagine | 2 TMS |
| AA | Aspartic acid | 2 TMS |
| | | 3 TMS |
| AA | Cysteine | 3 TMS |
| AA | Glutamine | 3TMS |
| AA | Glycine | 2TMS |
| | | 3TMS |
| AA | Isoleucine | 1TMS |
| | | 2TMS |
| AA | Leucine | 1TMS |
| | | 2TMS |
| AA | Lysine | 3TMS |
| AA | Methionine | 1TMS |
| | | 2TMS |
| AA | Phenylalanine | 1TMS |
| | | 2TMS |
| AA | Proline | 1TMS |
| | | 2TMS |
| AA | Serine | 2TMS |
| | | 3TMS |
| | | 4TMS |
| AA | Threonine | 2TMS |
| | | 3TMS |
| AA | Tryptophan | 2TMS |
| AA | Tyrosine | 3TMS |
| AA | Valine | 1TMS |
| | | 2TMS |
| Glycerol | Dihydroxyacetone phosphate | 1MeOX 3TMS |
| Glycerol | Glycerol | 3TMS |
| Glycerol | Glycerol-3-phosphate | 4TMS |
| Glycolysis | Fructose-6-phosphate | 1MeOX 6TMS |
| Glycolysis | Glucose-6-phosphate | 1MeOX 6TMS |
| Glycolysis | Glyceric acid-3-phosphate | 4TMS |

| | | |
|------------|---------------------------|------------|
| Glycolysis | Lactic acid | 2TMS |
| Glycolysis | Phosphoenolpyruvic acid | 3TMS |
| Glycolysis | Pyruvic acid | 1MeOX 1TMS |
| Nucleobase | Adenine | 2TMS |
| Nucleobase | Uracil | 2TMS |
| Nucleosid | Adenosine | 3TMS |
| Nucleosid | Adenosine | 4TMS |
| Nucleosid | Cytosine | 2TMS |
| Others | Butanoic acid, 3-hydroxy- | 2TMS |
| Others | Butanoic acid, 4-amino- | 3TMS |
| Others | Erythritol | 4TMS |
| Others | Glutaric acid | 2TMS |
| Others | Glyceric acid | 3TMS |
| PPP | Ribose-5-phosphate | 1MeOX 5TMS |
| PPP | Ribose | 1MeOX 4TMS |
| TCA | Citric acid | 4TMS |
| TCA | Fumaric acid | 2TMS |
| TCA | Glutamic acid | 2TMS |
| | | 3TMS |
| TCA | Glutaric acid, 2-hydroxy- | 3TMS |
| TCA | Glutaric acid, 2-oxo- | 1MeOX 2TMS |
| TCA | Malic acid | 3TMS |
| TCA | Succinic acid | 2TMS |

Table S8. Technical variation during gas chromatography mass spectrometry (GC-MS) run of four pooled samples. Alkane 32 (C₃₂H₆₆, Dotriacontane) is an acyclic saturated hydrocarbon containing 32 carbons. It is used to determine the instrument stability over time. Alkane 32 is measured independently of any extraction or derivatization steps. RSD: Relative standard deviation.

| | RSD |
|--------------------------|------------|
| Sum of area | 10% |
| Alkane 32 | 15% |
| Internal standard | 18% |

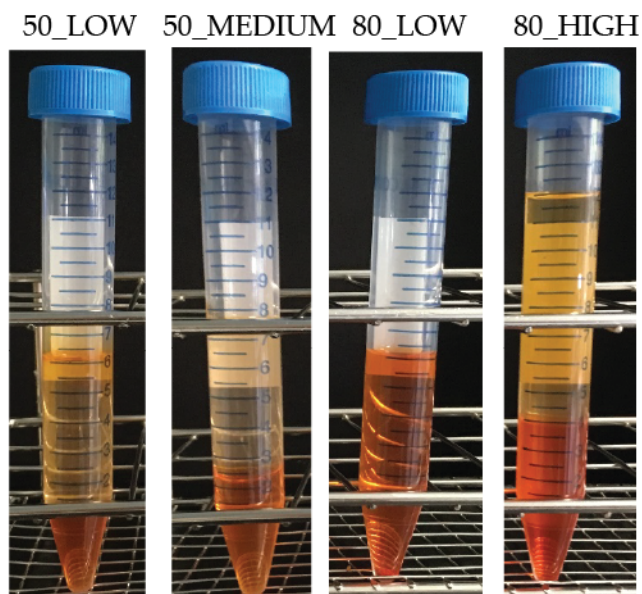


Figure S1. 50% and 80% of MeOH quenching buffer and different ratios of MeOH:CHCl₃:H₂O were mixed for extraction steps. Sudan I was added to aid visualization of phase separation. No phase separation could be observed using 80_LOW condition (1.0 mL CHCl₃ for extraction resulting in a final volume of 6.0 mL). Therefore, the condition was not used for further testing.

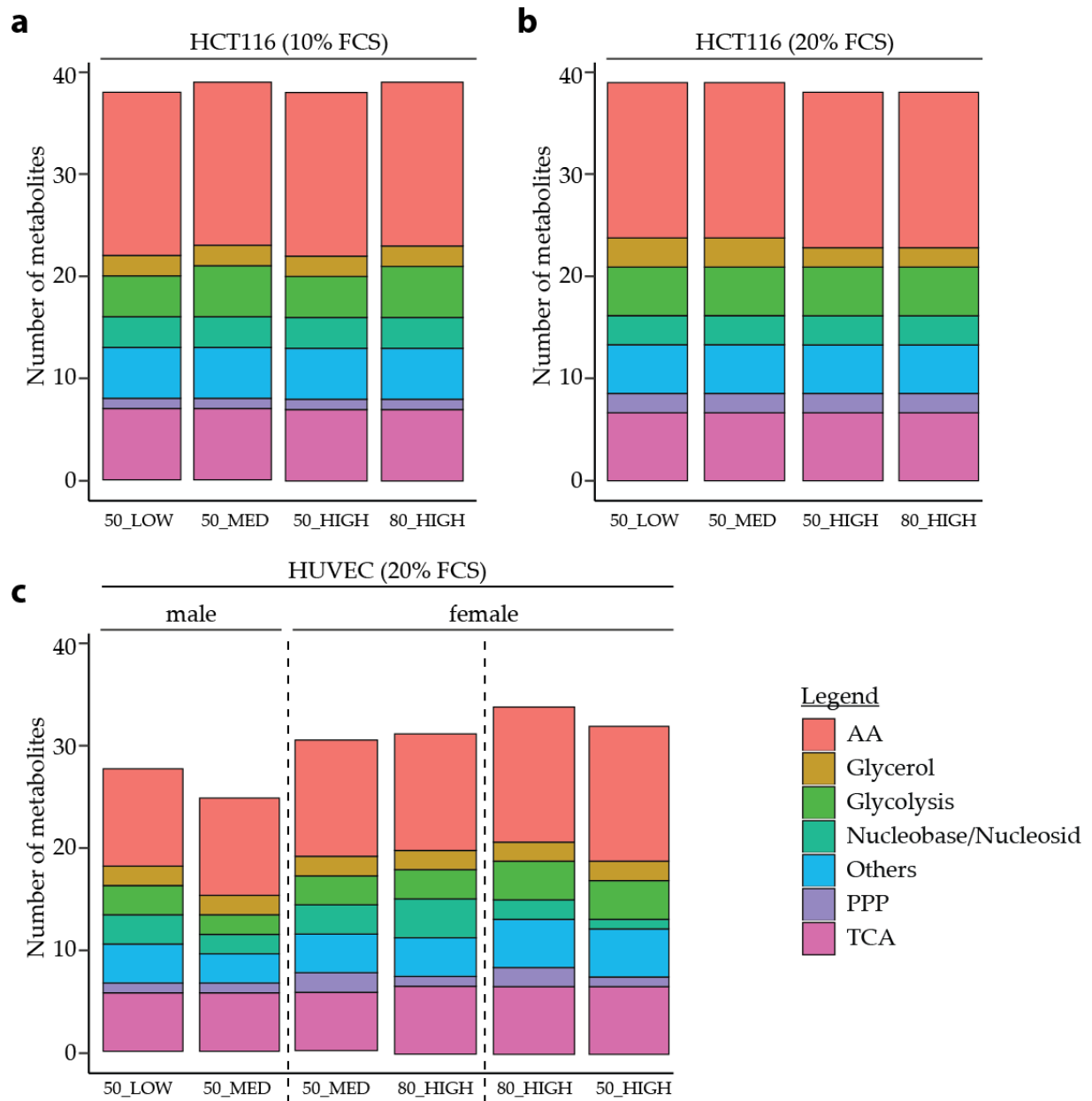


Figure S2. Recovery of annotated metabolites per biological group for different quenching and extraction methods. **(a)** HCT116 cells were cultivated in 10% FCS (in minimum three out of five biological replicates). **(b)** HCT116 cells were cultivated in 20% FCS (in minimum four out of five biological replicates). **(c)** HUVECs were cultured in 20% FCS (in minimum two out of three biological replicates). HCT116 cells were measured in one batch while HUVECs were measured in different batches (dashed line). The column data represents the number of annotated metabolites for each protocol. AA: Amino acids. PPP: Pentose phosphate pathway. TCA: Tricarboxylic acid cycle.

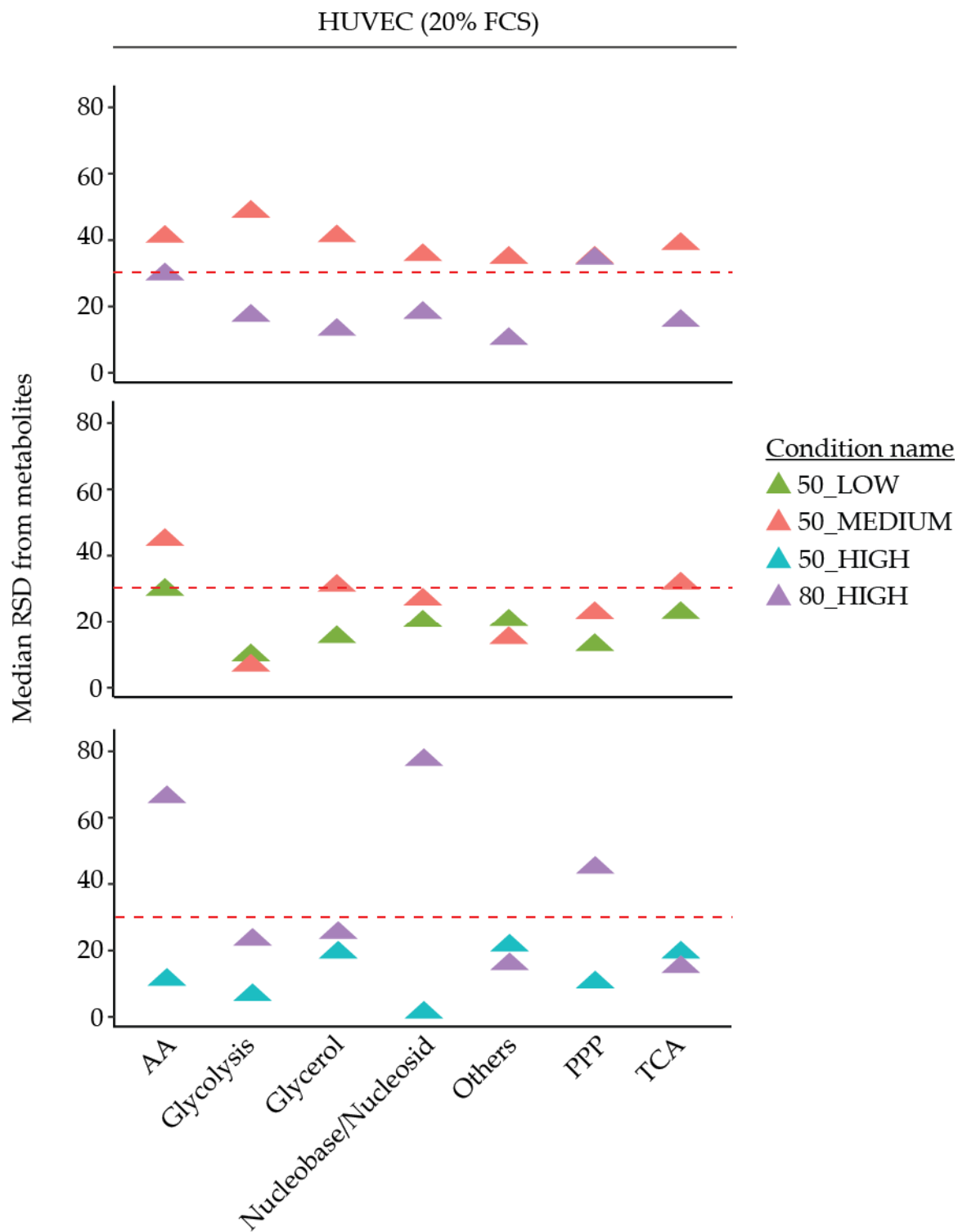


Figure S3. Median relative standard deviation (RSD) of individual metabolites separated by metabolite classes from in minimum two out of three replicates of HUVECs cultured in 20% FCS conditions using different quenching solvents, extraction ratios and polar volumes. The dashed line represents the maximum 30% RSD threshold advised by the Food and Drug Administration (FDA). Cells were measured in different batches reflected by the three graphs. AA: Amino acids. PPP: Pentose phosphate pathway. TCA: Tricarboxylic acid cycle.

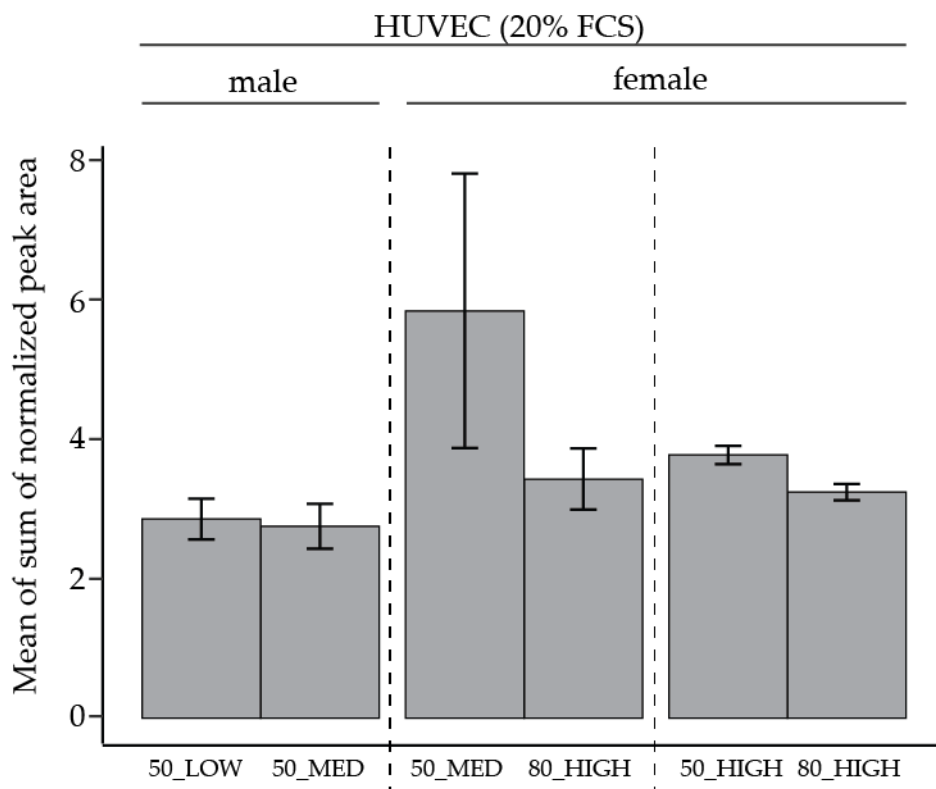


Figure S4. Comparison of the mean of sum of normalized peak area for different quenching and extraction methods. HUVECs were cultured in 20% FCS. The cells were measured in 2 different batches (dashed line). Data from in minimum two out of three biological replicates. The peak area was normalized to cinnamic acid and protein amount. Due to the low number of biological replicates no significances were measured.

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File S1. Standard operating protocol for harvest and extraction of adherent cells grown under 10% or 20% fetal calf serum (FCS) conditions version 1.

If you use this protocol please cite as: Fritsche-Guenther et al., "Modified protocol of harvesting, extraction and normalization approaches for gas chromatography mass spectrometry-based metabolomics analysis of adherent cells grown under high fetal calf serum conditions", *Metabolites* 2019

This protocol has been validated with respect to quenching buffers and extraction solvents using HUVEC and HCT116 cells. Other variables e.g. wash buffers, cell culture conditions, media changes etc. will be cell-type dependent and need to be determined by the user.

Researchers should familiarize themselves with the health and safety implications of the procedures and chemicals they will be using and take appropriate precautions as necessary in consultation with the Health and Safety department of their institution. Solvents should be GC-MS grade or higher. Prepare all necessary equipment and solvents in advance. Use the same buffers, solvents and batch of vials for any individual study.

To reduce the technical variability of this method, it is important that cells are harvested quickly and reproducibly using ice cold methanol (MeOH). To ensure these conditions, it is recommended that no more than three culture plates are harvested at a time.

Sample type

Adherent cell lines or primary cells plated in 10 cm² dishes. Confluency at day of harvest should be 80-90%.

Equipment (prepare in advance)

- Appropriate working area in accordance with your local health and safety guidelines e.g. fume hood or solvent safe microbiologic cabinet
- 15 mL Falcon (2 per cell culture plate)
- 5 mL pipette and tips (do not use a pipette controller or a pump for media removal)
- Crushed ice
- Cell scraper (uncover cell scraper prior to use and store in completely clean glass beaker)
- Centrifuge (cooled to 4°C)
- Rotational vacuum concentrator

Solvents

- **Washing buffer** (140 mM sodium chloride, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid = HEPES, pH 7.4) + supplements (depend on the growth media conditions) **pre-warmed to 37 °C**. Suggested maximum storage time for washing buffer is 2 months, however, check before experiment if the buffer is cloudy; if so, prepare new.
- **50% MeOH** in water (contains 2 µg/mL cinnamic acid) **pre-chilled to -20 °C**. When in use, the solution should be stored on ice to keep it cold. Maximum time the MeOH solution can be used should be 30 min to avoid it warming.
- 100% MeOH
- Chloroform (CHCl₃)
- Water (H₂O)

Protocol

Harvest

1. Wash cells rapidly (20 seconds) with 5 mL of washing buffer.
2. Quench cells by using 5 mL of ice-cold 50% MeOH.
3. Immediately scrape cells into the MeOH solution and collect the methanolic lysates into a 15 mL Falcon. Samples can be stored at -80 °C until extraction.

Extraction

4. For extraction add 4 mL of CHCl₃, 1.5 mL of 100% MeOH and 1.5 mL of H₂O to the methanolic cell extracts.
5. Shake extracts for 60 min at 4 °C.
6. Centrifuge extracts at 4,149 × g for 10 min at 4 °C to separate the phases.
7. Collect 6 mL of the upper polar phase and transfer to a new 15 mL Falcon.
8. Create a pooled quality control (QC) sample by pooling the leftover polar phase from each sample. The amount of pooled QC required is dependent on the number of samples to be analysed and your QC regime (recommended two at the beginning of the run, two at the end and every 5 to 10 samples). The pooled QC should then be divided into 6 mL aliquots for further extraction. 6 mL is enough to create two QC samples. This pooled sample is then extracted alongside the other samples and used as quality control for technical variability of the extraction process and instrument.

OPTIONAL: if you wish to measure total protein content, keep the original Falcon tube and follow steps 10 to 14.

9. Dry the polar phase at 30 °C at a speed of 1,550 × g at 0.1 mbar using a rotational vacuum concentrator. Samples can be stored at -80 °C until backup generation or GC-MS measurement.

OPTIONAL: To generate backup samples (and to generate two QCs from a single extraction), samples can be split before being dried down, or can be resuspended and split after drying down. The latter method can have some advantages if you are limited in space in your rotational vacuum concentrator.

Measurement of total protein

10. The original Falcon tube should now have a protein pellet and a CHCl₃ mix left. The total protein amount can be measured from this if required for normalization. If this is required, add 8 mL of 100% MeOH to what remains in the Falcon tube.
11. Vortex for 10 sec and centrifuge at 16,000 × g for 10 min.
12. Carefully discard the supernatant.
13. Air dry the pellet at room temperature for 30 min.
14. Resuspend the pellet in an appropriate buffer and determine the protein amount using your favored method. We resuspend the pellet in 8 M urea buffer (in 50 mM HEPES, pH 8.5) and determined the protein concentration using a bicinchoninic acid (BCA) assay (Thermo Fischer Scientific, Waltham, Massachusetts, US) following the manufacturer's instructions.