Uncertainty budgeting in fold change determination and implications for non-targeted metabolomics studies in model systems

Karin Ortmayr^{*a,b*}, Verena Charwat^{*c*}, Cornelia Kasper^{*c*}, Stephan Hann^{*b*}, Gunda Koellensperger^{*a**}

^a Institute of Analytical Chemistry, University of Vienna, Faculty of Chemistry, Waehringer Strasse 38, 1090 Vienna, Austria

^b Department of Chemistry, University of Natural Resources and Life Sciences (BOKU) Vienna, Muthgasse 18, 1190 Vienna, Austria

^c Department of Biotechnology, University of Natural Resources and Life Sciences (BOKU) Vienna, Muthgasse 18, 1190 Vienna, Austria

KEYWORDS

Non-targeted metabolomics – Fold change – Uncertainty budgeting – Significance testing – Twodimensional liquid chromatography – Human mesenchymal stem cells

SUPPLEMENTARY INFORMATION

Input quantities \rightarrow		A ₁	A ₂		
\checkmark	Values of input quantities	Metabolite intensity in group 1	Metabolite intensity in group 2		
Associated standard uncertainties	-	0.22 x 10 ⁴ (15% CV)	0.21 x 10 ⁴ (7% CV)		
A ₁	1.45 x 10 ⁴	1.67 x 10 ⁴	1.45 x 10 ⁴		
A ₂	3.10×10^4	3.10×10^4	3.31×10^4		
$FC = \frac{A_2}{A_1}$	2.138	1.856	2.283		
Dev	-	0.282	-0.145		
Dev ²	-	0.079	0.021		
Dev ² _{sum}	0.100	-	-		
u _{FC}	0.32	Total combined uncertainty			
U _{FC} (k = 2)	0.63	Expanded uncertainty			
U _{FC} (relative)	30%	Relative expanded uncertainty			
Reported FC	2.14 ± 0.63				

Table S1. Example for the calculation of the expanded uncertainty associated with a given fold change, following the Kragten spreadsheet method²⁸.

Table S2. List of 58 metabolites contained in the standard-based QC sample and their retention times in RP-PGC-TOFMS. Retention times are average values across replicate injections of the QC sample (n = 5 technical replicates) throughout the total measurement time in positive or negative ionization mode. Further information about the selectivity and metabolite coverage of this method is available in K. Ortmayr, S. Hann and G. Koellensperger, *Analyst*, 2015, **140**, 3465–3473 [ref. 38].

Abbr.	Metabolite	Polarity	Exact mass, m/z	Retention time, min	Retention time RSD (<i>n</i> = 5)
Aco	cis-Aconitate	-	173.0090	5.55	0.8%
Ade	Adenine	+	136.0618	2.59	0.2%
ADP	Adenosine diphosphate	-	426.0221	3.16	0.4%
AKG	α-Ketoglutarate	-	145.0142	3.20	0.6%
Ala	Alanine	+	90.0550	3.56	0.2%
3AMP	3'-Adenosine monophosphate	-	346.0558	5.55	0.9%
5AMP	5'-Adenosine monophosphate	-	346.0558	3.39	0.4%
Arg	Arginine	+	175.1190	3.30	0.4%
Asin	Adenosine	+	268.1040	5.51	0.7%
Asn	Asparagine	+	133.0608	3.71	0.3%
Asp	Aspartate	+	134.0448	4.22	0.7%
Cit	Citrate	-	191.0197	3.87	0.5%
Cysta	Cystathionine	+	223.0747	3.83	0.2%

Abbr.	Metabolite	Polarity	Exact mass, m/z	Retention time, min	Retention time RSD (<i>n</i> = 5)
FAD	Flavinadenine dinucleotide	+	786.1644	7.71	0.9%
Fum	Fumarate	-	115.0037	4.66	0.9%
Glc-On	Gluconate	-	195.0510	6.82	1.1%
Gln	Glutamine	+	147.0764	3.93	0.4%
Glu	Glutamate	+	148.0604	4.26	0.4%
Gly	Glycine	+	76.0393	3.46	0.3%
GMP	5'-Guanosine monophosphate	-	362.0507	4.05	0.6%
Gnin	Guanine	+	152.0567	2.90	0.1%
GSH	Glutathione, reduced	+	308.0911	3.38	0.1%
Gsin	Guanosine	+	284.0989	5.71	0.8%
GSSG	Glutathione, oxidized	+	613.1592	5.5	0.4%
His	Histidine	+	156.0768	3.29	0.2%
I-Cit	Isocitrate	-	191.0197	2.60	0.2%
lle	Isoleucine	+	132.1019	4.66	0.3%
Kile	Ketoisoleucine	-	129.0557	8.93	0.8%
Lac	Lactate	-	89.0244	3.09	0.2%.
Leu	Leucine	+	132.1019	5.10	0.4%
Lys	Lysine	+	147.1128	2.54	0.2%
Mali	Malate	-	133.0142	9.06	0.3%
Man-Ol	Mannitol	+	183.0863	4.30	0.4%
Met	Methionine	+	150.0583	3.05	0.2%
NAD^+	Nicotinamide adenine	+	664.1164	5.13	0.8%
$NADP^+$	Nicotinamide adenine	+	744.0827	3.91	0.2%
PEP	Phosphoenolpyruvate	-	165.9673	19.15	0.8%
2PG	2-Phosphoglycerate	-	184.9857	11.05	0.4%
3PG	3-Phosphoglycerate	-	184.9857	11.54	0.4%
6PGA	6-Phosphogluconate	-	275.0174	13.5	0.4%
Phe	Phenylalanine	+	166.0863	6.44	0.5%
Pro	Proline	+	116.0706	4.10	0.2%
Pyr	Pyruvate	-	87.0088	2.62	0.5%
R5P	Ribose 5-phosphate	-	229.0119	10.8	0.4%
Ri-Fl	Riboflavin	+	377.1456	8.08	0.6%
RI5P	Ribulose 5-phosphate	-	229.0119	11.2	0.4%
S7P	Sedoheptulose 7-phosphate	-	289.0330	11.7	0.3%
SAH	S-Adenosylhomocysteine	+	385.1289	5.50	0.4%
Ser	Serine	+	106.0499	3.56	0.2%
Suc	Succinate	-	117.0193	4.97	0.6%
Thr	Threonine	+	120.0655	3.69	0.2%
Thy	Thymine	+	127.0502	5.79	0.5%

Abbr.	Metabolite	Polarity	Exact mass, m/z	Retention time, min	Retention time RSD (<i>n</i> = 5)
Trp	Tryptophan	+	205.0972	7.17	0.5%
Tyr	Tyrosine	+	182.0812	4.99	0.4%
5UMP	5'-Uridine monophosphate	-	323.0286	3.13	0.4%
Ura	Uracil	+	113.0346	3.14	0.3%
Uri	Uridine	+	245.0768	5.50	0.4%
Val	Valine	+	118.0863	4.65	0.1%

Table S3 Stem cell culture parameters.

	Normoxia	Нурохіа
Seeding cell count (n = 3)	$(2.30 \pm 0.05) \times 10^{6}$	$(2.30 \pm 0.05) \times 10^{6}$
Final cell count ($n = 3$)	$(1.06 \pm 0.11) \times 10^{6}$	(1.10 ± 0.05) x 10 ⁶
Population doublings	2.20	2.25
Doubling time, h	21.7	21.3
Total protein content ($n = 5$), µg per well	260 ± 10	258 ± 8
Glucose concentration in cell- free medium ^a , $mg \cdot L^{-1}$	968 ± 12	985 ± 1
Residual glucose in culture supernatant ^b , $mg \cdot L^{-1}$	27.3 ± 8.7	5.2 ± 4.7
Lactate concentration in cell-free medium ^a , $mg \cdot L^{-1}$	25.6 ± 1.7	28.0 ± 0.5
Lactate concentration in culture supernatant ^b , $mg \cdot L^{-1}$	798 ± 37	801 ± 85

 $^{\rm a}$ Aliquots of the medium were incubated without the addition of cells for 48 h, n = 3.

^b Culture supernatants were sampled after 48 h, immediately before cell harvest and metabolite extraction, n = 3.

Table S4. Expanded uncertainty and minimum significant FC values for the 31 compounds identified as statistically significant in a moderated t-test ($p_{corr} < 0.05$, Benjamini-Hochberg FDR) for the stem cell data set. Fold changes (FC) are calculated for the hypoxic condition (H) relative to normoxic condition (N). Uncertainties (U_{FC}) are given as relative expanded uncertainty (k = 2). FC_{min} is calculated according to (Eq. 6) and represents the smallest FC value distinguishable from FC = 1 within uncertainty. Compounds where the observed FC does not exceed FC_{min} (i.e. should not be considered relevant) are marked in grey. Coefficients of variation (CV) are shown after sample-specific scaling to the total protein content for n = 5 biological replicates.

	FC	<i>p</i> -value	CV			FC _{min}
		(corr.)	Ν	н		
Compound 1	-1.49	0.002	4%	11%	15%	1.17
Compound 2	-1.48	0.002	3%	10%	14%	1.16
Compound 3	-1.43	0.002	3%	6%	9%	1.09
Compound 4	-1.34	0.034	15%	8%	22%	1.29
Compound 5	-1.29	0.008	7%	3%	12%	1.13
Compound 6	-1.20	0.029	11%	3%	17%	1.21
Compound 7	-1.16	0.020	2%	3%	6%	1.07
Compound 8	1.14	0.034	6%	3%	14%	1.16
Compound 9	1.15	0.045	4%	4%	13%	1.16
Compound 10	1.15	0.028	4%	3%	10%	1.12
Compound 11	1.15	0.029	3%	2%	9%	1.10
Compound 12	1.15	0.045	4%	3%	12%	1.14
Compound 13	1.16	0.045	5%	5%	16%	1.20
Compound 14	1.17	0.029	3%	6%	14%	1.17
Compound 15	1.18	0.029	4%	2%	11%	1.13
Compound 16	1.18	0.029	5%	6%	19%	1.24
Compound 17	1.19	0.047	10%	8%	29%	1.41
Compound 18	1.19	0.029	4%	5%	16%	1.18
Compound 19	1.20	0.018	5%	4%	16%	1.19
Compound 20	1.20	0.029	7%	7%	23%	1.30
Compound 21	1.21	0.029	8%	9%	28%	1.39
Compound 22	1.23	0.045	9%	7%	27%	1.36
Compound 23	1.24	0.008	4%	2%	11%	1.13
Compound 24	1.28	0.020	4%	10%	27%	1.37
Compound 25	1.32	0.025	11%	11%	38%	1.62
Compound 26	1.34	0.029	5%	15%	42%	1.72
Compound 27	1.35	0.004	8%	8%	30%	1.42
Compound 28	1.37	0.002	9%	6%	29%	1.41
Compound 29	1.41	0.005	11%	10%	39%	1.63
Compound 30	1.46	0.004	12%	7%	37%	1.58
Compound 31	1.48	0.002	7%	10%	35%	1.53



Figure S1. Assessing the variability of fold change (FC) values using two different approaches. Results are shown for the 248 unknown compounds that passed all quality filters applied to the stem cell data set (n = 5 biological replicates in both groups), evaluated using a non-targeted metabolomics workflow in MassHunter Profinder and MassProfiler Professional 13.1 (both by Agilent Technologies, Santa Clara, CA, USA). **a)** Uncertainty propagation approach, error bars reflect the expanded uncertainty of the FC values calculated from group-averaged peak areas. **b)** Bootstrap resampling approach, error bars reflect 95% confidence intervals as determined in bootstrap resampling (unordered sampling with replacement of 5 out of 5 values per group, 100 iterations).