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Isotope-assisted metabolomics for the quantification of plasma metabolites: A preliminary inter-laboratory trial



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WHY?

Human large-scale metabolomics analysis is facing important challenges to increase its robustness, reproducibility and interoperability. In this context, **stable-isotope labeled compounds** are increasingly used for **identification and quantification** of endogenous metabolites.

HOW?

In this study, two laboratories from the **MetaboHUB consortium** were involved in an **inter-laboratory absolute quantification trial** of **amino and organic acids** in the reference human plasma sample (**NIST-SRM-1950**) using 24 commercially-available stable-isotope (¹³C / ¹⁵N / D) labeled standards, among the 416 Level 1 annotated¹ metabolites in the MetaboHUB consortium.

A pool of the labeled compounds was prepared in concentrations similar to what is expected in human plasma from the literature² (**Table 1**). Labeled compounds were selected with a molecular weight difference higher than 3, compared to their endogenous homologs, to avoid overlapping with the signal of the naturally occurring isotopomers.

Table 1. Description of the labeled compounds used for the preliminary quantification trial

Metabolite	HMDB blood concentration range (μM) ^a	[NIST] (μM ± S.E.M) ^{b,2}	[Pool] (μM)	Labeling	MW (Da) endogenous	Δ MW (Da)
L-alanine	100-500	258 ± 17	310	¹³ C ₃	89.05	3.01
L-arginine	40-218	69 ± 4	69	¹⁵ N ₄	174.11	3.99
L-asparagine	23-87	37.3 ± 1	56	¹³ C ₄	132.05	4.01
L-aspartic acid	6-70	1.2 ± 0.1	2	¹³ C ₄ , ¹⁵ N	133.04	5.01
L-citrulline	10-70	18 ± 1.5	18.8	4, 4, 5, 5-D ₄	175.10	4.03
creatine	30-84	32 ± 2	28	Me D ₃	131.07	3.02
creatinine	30-82	60 ± 2	56	N-Methyl-D ₃	113.06	3.02
glycine	106-272	215 ± 7	205	¹³ C ₂	75.03	2.01
L-histidine	22-108	60 ± 1	60	¹⁵ N ₃	155.07	2.99
L-methionine	4-40	31.7 ± 1	33	Me D ₃	149.05	3.02
L-phenylalanine	16-71	54.8 ± 1.4	53	RING- ¹³ C ₆	165.08	6.02
L-glutamic acid	8-80	76 ± 3	73	¹³ C ₅	147.05	5.02
L-glutamine	380-780	460.4 ± 18	472	¹³ C ₅	146.07	5.02
L-serine	62-161	99 ± 5	100	¹³ C ₃	105.04	3.01
L-threonine	43-300	113 ± 4	103	¹³ C ₄ , ¹⁵ N	119.06	5.01
L-tryptophan	20-80	48 ± 0.8	49	INDOLE-D ₅	204.09	5.03
L-tyrosine	24-105	57.3 ± 0.9	55	¹³ C ₉ , ¹⁵ N	181.07	10.03
succinic acid	8-23	3 ± 0.2	2.9	¹³ C ₄	118.03	4.01
(S)-3-hydroxybutyric acid	36-260	260 ± 16	245	¹³ C ₄	104.05	4.03
citric acid	30-400	191/268	198	2,2,4,4-D ₄	192.03	4.03
fumaric acid	0-4	11 ± 0.11	9.9	¹³ C ₄	116.01	4.01
(S)-malic acid	3.2-12	0.9 ± 0.1	2.1	2,3,3-D ₃	134.02	3.02
L-lactic acid	450-2200	n/a	505	¹³ C ₃	90.03	3.01
2-oxoglutaric acid	7.0-20.7	19 ± 0.7	19.1	¹³ C ₅	146.02	5.02

^aConcentration ranges in human blood compiled in HMDB (see Ref. 2); ^bThree technical replicates every 4 batches (see Ref. 2)

The labeled pool, the NIST-SRM-1950 and the spiked NIST-SRM-1950 were analyzed by LC-HRMS using both C18 and HILIC chromatography (**Table 2**).

Table 2. Protocol descriptions of plasma metabolite extraction and LC-HRMS measurement in both laboratories (a) MetaboHUB Clermont-Ferrand (MTH-Clermont) & (b) MetaboHUB Ile-de-France (MTH-IDF)

Laboratory	(a) MTH-Clermont	(b) MTH-IDF	(b) MTH-IDF
Metabolite extraction	100 μL plasma NIST-SRM-1950 30 min at -20°C → 150 μL supernatant	100 μL plasma NIST-SRM-1950 1h 30 min in ice → all supernatant	400 μL cold methanol (-20°C)
Solubilization solvent(s)	500 μL 50/50 water/acetonitrile + 0.1% HCOOH	300 μL 95/5 water/acetonitrile + 0.1% HCOOH	120 μL (NH ₄) ₂ CO ₃ 10 mM – pH 10.5* + 180 μL acetonitrile
LC	Thermo Scientific UHPLC U3000	Thermo Scientific UHPLC U3000	
Column description / T(°C)	C18 HSS T3 150 x 2.1 mm x 1.8 μm Waters / 30°C	C18 Hypersil Gold 150 x 2.1 mm x 1.9 μm Thermo / 30°C	SeQuant® Zic-p-Hilic 150 x 2.1 mm x 5 μm Merck / 15°C
Mobile Phase	A: water + 0.1% HCOOH B: acetonitrile + 0.1% HCOOH	A: water + 0.1% HCOOH B: acetonitrile + 0.1% HCOOH	A: (NH ₄) ₂ CO ₃ 10 mM – pH 10.5* B: acetonitrile
Run time / Flow rate / Injection volume	26 min / 0.4 mL.min ⁻¹ / 5 μL	30 min / 0.5 mL.min ⁻¹ / 10 μL	42 min / 0.2 mL.min ⁻¹ / 10 μL
Step gradient	2min: 0% B; 13 min: 0 to 100% B; 7 min: 100% B; 0.1 min: 100 to 0% B; 3.9 min: 0% B	2min: 5% B; 11 min: 5 to 100% B; 12.5 min: 100% B; 0.1 min: 100 to 5% B; 4.4 min: 5% B	2min: 0% B; 10 min: 80 to 40% B; 0.1 min: 40 to 0% B; 4.9 min: 0% B; 0.1 min: 0 to 80% B; 24.9 min: 80% B
HRMS	QTOF, Bruker Impact II / ESI mode: POS & NEG / Scan range: 50 to 1000 m/z	QTOF, Bruker Impact HD / ESI mode: POS & NEG / Scan range: 50 to 500 m/z	Orbitrap, Fusion / ESI mode: POS & NEG / Scan range: 70 to 1000 m/z
Data processing	Skyline	Data Analysis	XCalibur

* adjusted with NH₄OH 28%

AS A RESULT...

Regardless of chromatography or detection mode, 19/24 endogenous metabolites and their labeled homologs were detected without any effects of labeling on retention times of metabolites analyzed in buffer (**Figure 1A & 1B**) and in matrix (**Figure 1C & 1D**).

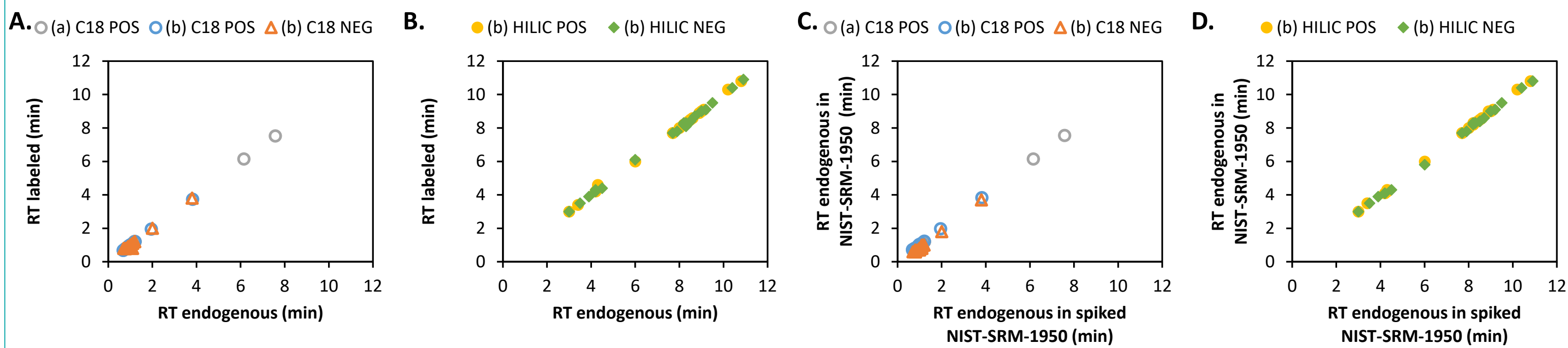


Figure 1: Scatter plot of retention time (RT) of labeled compounds vs endogenous metabolites in the spiked NIST-SRM-1950 obtained from **A. C18** & **B. HILIC** chromatography in both positive and negative modes

MS-peak areas of endogenous metabolites, including the m in spiked NIST-SRM-1950 were not impacted by the isotope addition (**Figure 2**).

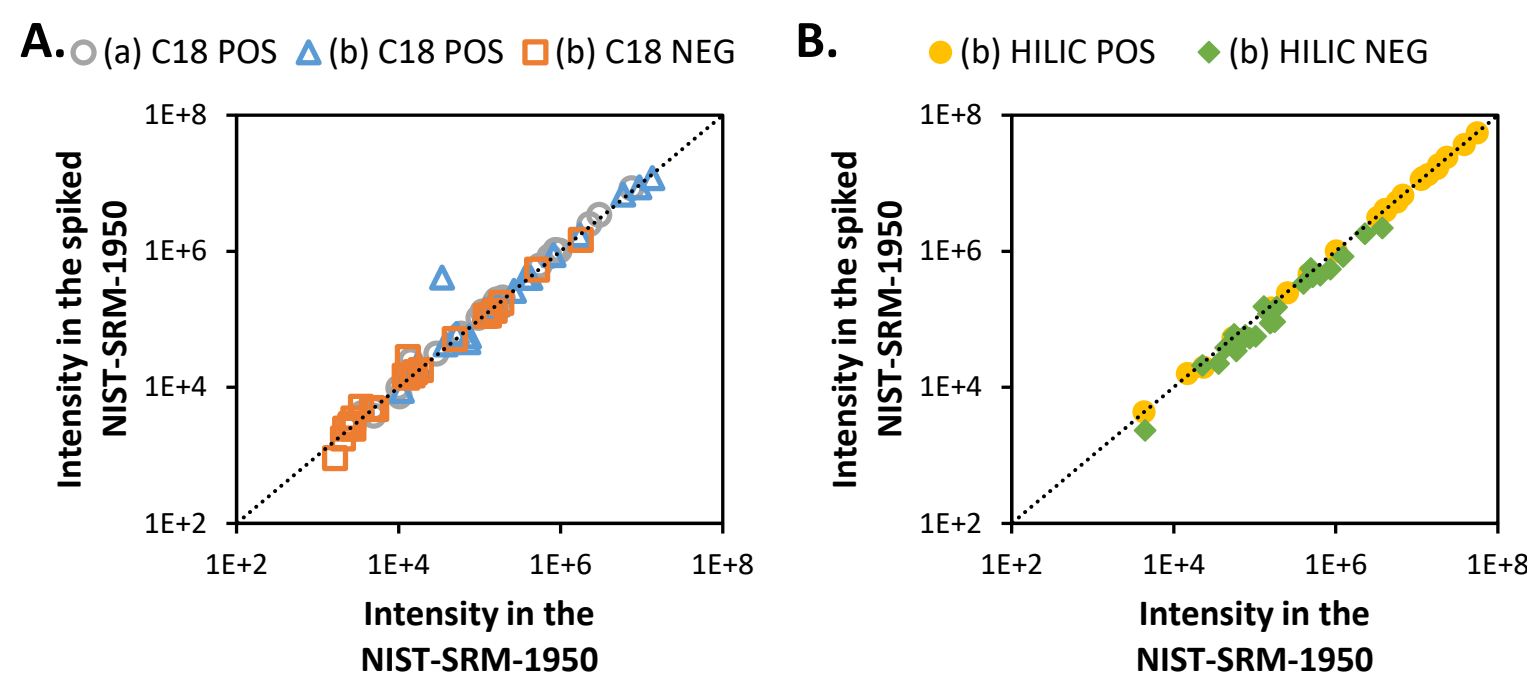


Figure 2: Scatter plot of intensity of endogenous metabolites in the NIST-SRM-1950 vs in the spiked NIST-SRM-1950 obtained from **A. C18** & **B. HILIC** chromatography in both positive and negative modes

Compared to the analysis in buffer, the intensity of the labeled compounds obtained from C18 was impacted by the dilution in plasma: a matrix effect accounted for an intensity attenuation > 1 log was observed for half of the selected metabolites (**Figure 3A**), generally those having an RT < 1 min. The selected compounds were more retained by the HILIC chromatography (RT: 2 to 12 min) which explains why matrix effect was not observed in this case (**Figure 3B**).

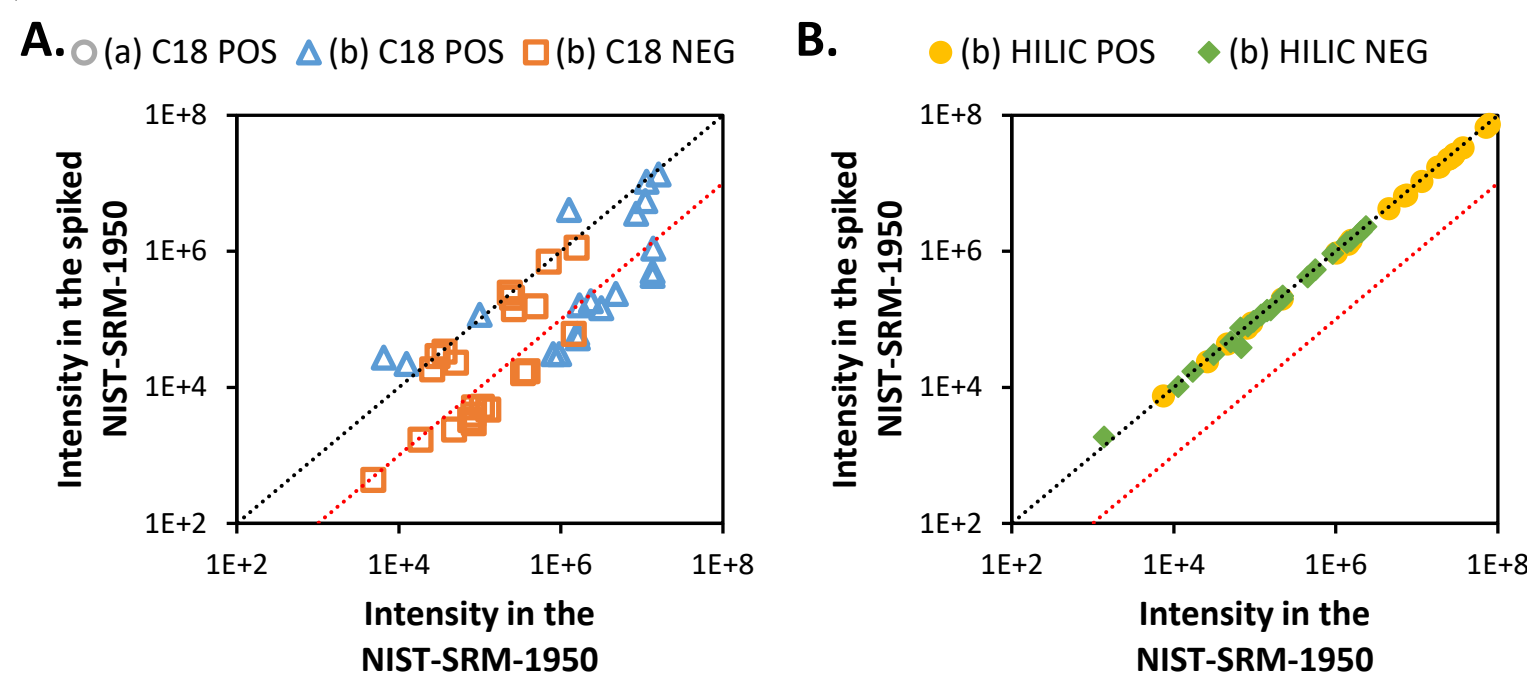


Figure 3: Scatter plot of intensity of labeled metabolites in the NIST-SRM-1950 vs in the spiked NIST-SRM-1950 obtained from **A. C18** & **B. HILIC** chromatography in both positive and negative modes

Results obtained with C18 POS were consistent between the two laboratories (a & b) (**Figure 4A**). Calculated absolute concentrations of endogenous metabolites were in accordance (±30%) with expected values (**Table 1**) for (a) 7 detected metabolites (out of 19) analyzed in C18 POS, and 4 metabolites (out of the 22) analyses in HILIC NEG (**Figure 4B**).

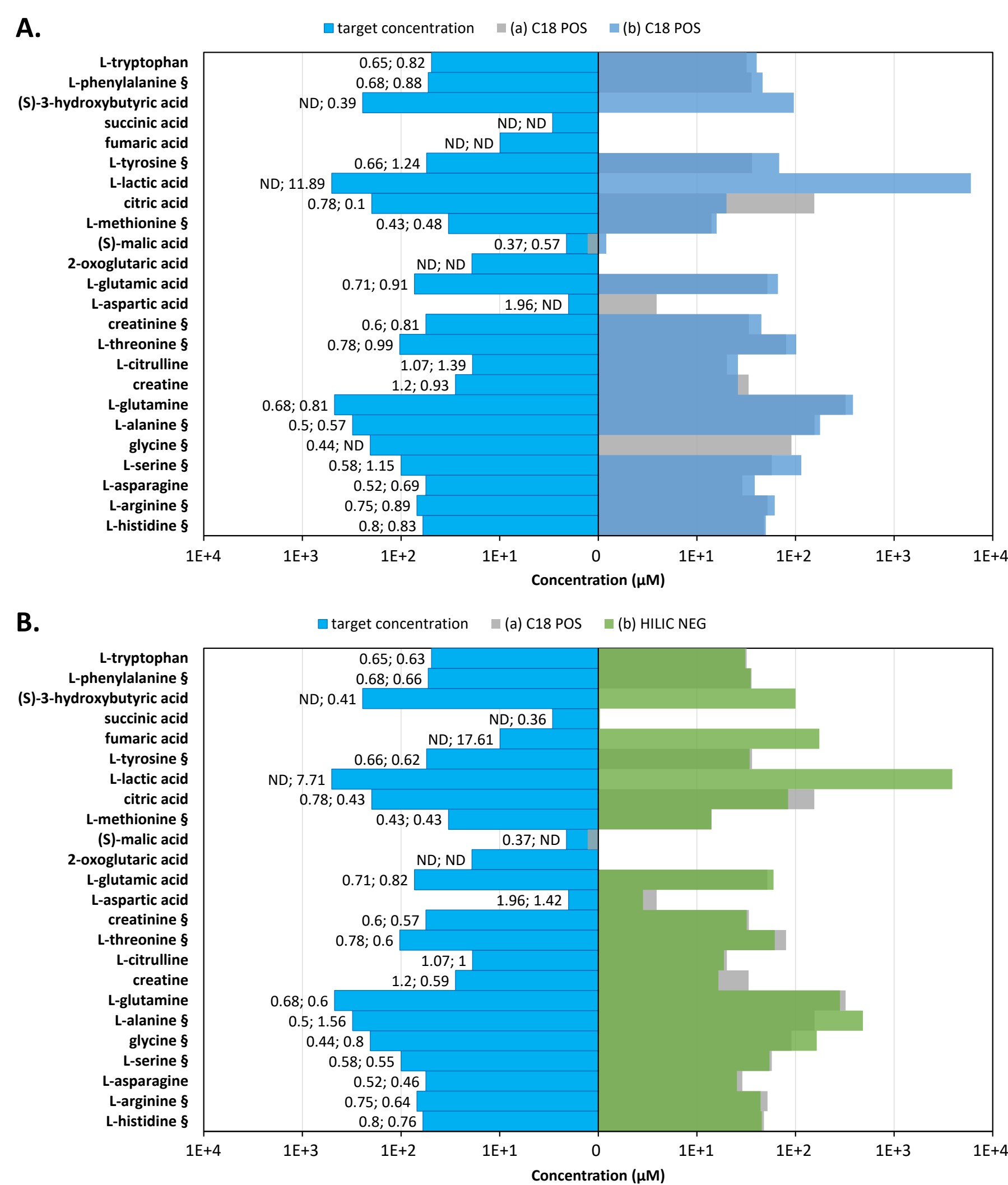


Figure 4: Target concentration of labeled compounds (left) vs absolute concentration of endogenous metabolites in the spiked NIST-SRM-1950 (right) calculated from the ratio of endogenous-to-labeled intensity (values in the center) obtained from **A. C18** & **B. HILIC** chromatography in both positive and negative modes (§ indicates concentration values in accordance with the NIST certificate)

TO CONCLUDE... This preliminary trial demonstrated that isotope-assisted metabolomics could provide comparable and reliable results across laboratories.

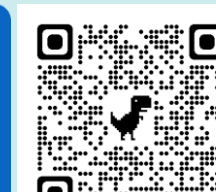
WHAT'S NEXT?

This inter-laboratory trial will be continued with some perspectives already set: (i) extend the list of metabolites to cover wider ranges of polarity and molecular weight with the possibility of using in-house synthesized labeled compounds, (ii) use biologically labeled compounds from yeast cultures, (iii) include in the trial more laboratories and platforms within the MetaboHUB consortium.

References: [1] Sumner et al. 2007 *Metabolomics*, 3: 211-221; [2] Liu et al. 2020 *Anal. Chem.*, 92: 8836-8844

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