Method for simplified simultaneous quantitation of the constituents of a chemically complex mixture and establishing quantitative linearity

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OVERVIEW

- Stable isotope labeled Internal Standards (IS) are key for accurate LC-MS metabolomics quantitation [1]. When analyzing a small number of compounds in a targeted protocol, it may be more accurate to quantify compounds individually, but when the extracts are biochemically complex, it becomes neither efficient nor economical.
- We have developed a simple workflow to efficiently quantify hundreds of metabolites in a biological extract. The workflow is suitable for both isotopic and natural abundance (NA) mixtures and sufficiently accurate for most purposes.
- A complex NA mixture is made, in which the concentration of every compound is known to establish the concentration of the same compounds in an IS mixture. The now quantified IS mixture is subsequently used to quantify unknown samples.
- In this study we examine the linearity and range of this technique.

METHODS

- In this method we used a single plate of 84 authentic from a 600 compound library (unlabeled, IROA-LSMLS) [2] that was derived from an accurately prepared mother liquors and formatted in seven 96-well plates, each well containing 1 mg of compound dissolved in 200 µL H2O and 50 µL IPA (Figure 2).
- We pooled 30 µL per well to create a solution that is serially diluted, aliquoted and dried. A fixed concentration of isotopically-labeled IS was added to all dried aliquots. Samples were analyzed using an Orbitrap Velos Pro mass spectrometer interfaced with Accela 1250 UHPLC system (Thermo Scientific). Metabolites were separated on an Ace Excel2 C18-PFP column. Apical ratios were used to calculate equivalent measurements for each compound.

WORKFLOW B (FIGURE 2)

- Each compound in the library had been previously analyzed to determine its RT. These identification parameters were loaded into a database to establish compound identity for subsequent analysis.
- The pooled serially diluted solutions containing IS were analyzed using our standard LC methodology (Figure 1). Using the database library peaks were identified and examined for their NA and IS isotopolog clusters using ClusterFinder™ software. An R program was written to perform the subsequent analysis.
- The apical ratio areas (12C/13C) were calculated and used to establish their point of equality. Since the concentrations of the 12C side were known (according to its aliquot size) therefore, the point at which the ratio was equal to 1 was used to the quantify the compounds on the IS (13C) side.
- Due to differences in the isotopic balance the ratio had to use all the isotopolog peaks for each of the NA and IS clusters in order to be accurate.
- The analysis was performed in both +ve and -ve ionization mode. This served as a secondary validation for some compounds.

Conflict of interest: Chris Beecher and Felice de Jong are co-founders of IROA Technologies

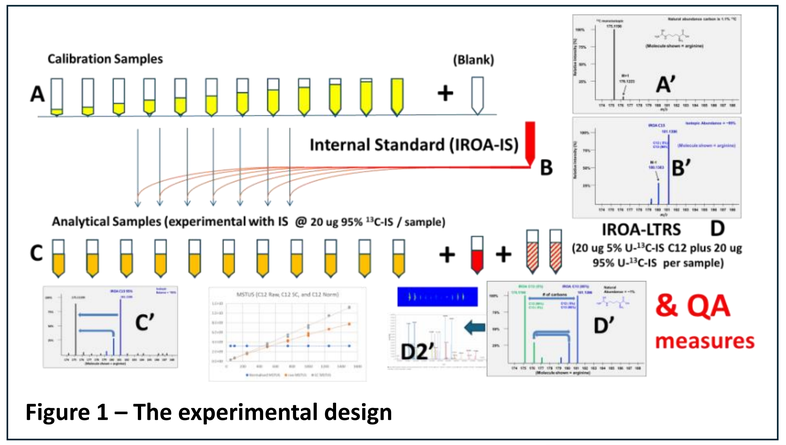

Figure 1 – The experimental design

Dilution label	Amount injected ug/ 5 ul	Concentration ug/40 ul	Aliquot size
L01	0.00019	0.00150	1
L02	0.00038	0.00300	2
L03	0.00075	0.00600	4
L05	0.00300	0.02400	16
L06	0.00600	0.04800	32
L07	0.03000	0.24000	160
L08	0.06000	0.48000	320
L09	0.15000	1.20000	800
L10	0.30000	2.40000	1600
L11	0.45000	3.60000	2400
L13	0.90000	7.20000	4800
L14	1.08000	8.64000	5760
L14	1.08000	8.64000	5760
L15	1.35000	10.80000	7200

Figure 3 – Concentrations in the gradient samples.

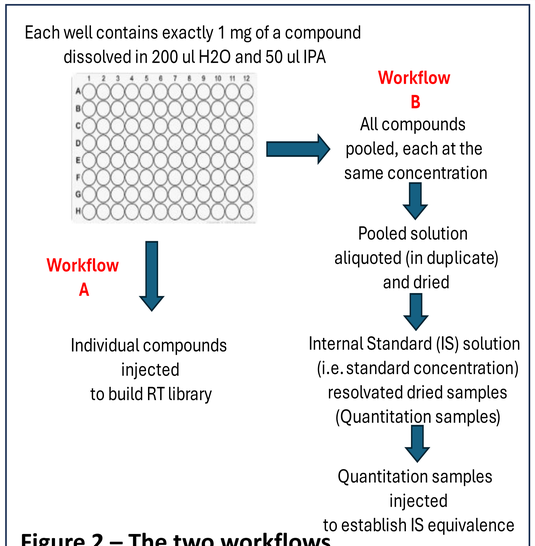

Figure 2 – The two workflows

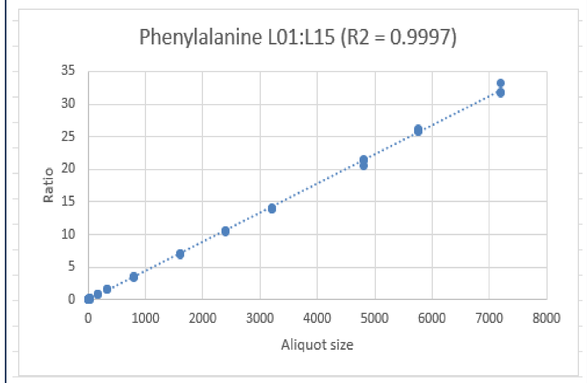

Figure 4 – The ratio between the natural abundance compound and the internal standard peaks is linear over a range from a 1X to 7200X aliquots, where each aliquot represents 0.0002 ug/ul.

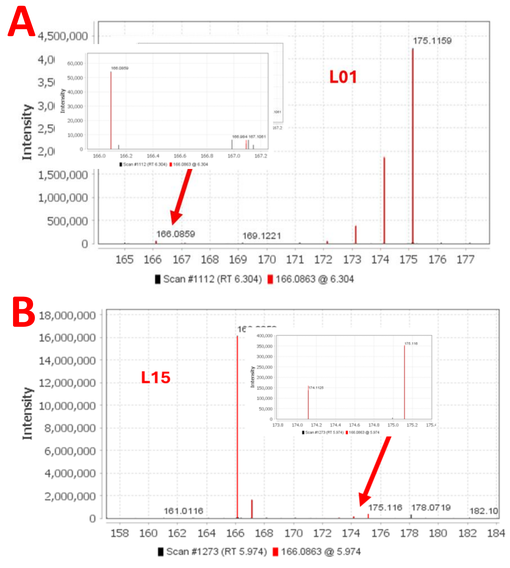

Figure 5 – Figure 5 – Quantitation is feasible if C12 and IS peaks are clearly detected (3X above noise, see A & B). Low-end ratios (<0.01) show significant error, while upper-end peaks (>1 to 30+) have minor errors.

C

label	Quant injected ug	amt/40 u	aliquot	aliquot	LTRS_000757 BasePos ratio	implied 1:1 ug	predicted values	error
L01	0.00019	0.00150	1	0.0090	0.02093	0.0050		44.2%
L01	0.00019	0.00150	1	0.0083	0.02251	0.0050		40.0%
L01	0.00019	0.00150	1	0.0087	0.02168	0.0050		42.2%
L02	0.00038	0.00300	2	0.0131	0.02854	0.0100		24.0%
L02	0.00038	0.00300	2	0.0127	0.02957	0.0100		21.2%
L02	0.00038	0.00300	2	0.0123	0.03041	0.0100		19.0%
L03	0.00075	0.00600	4	0.0198	0.03786	0.0200		-0.9%
L03	0.00075	0.00600	4	0.0202	0.03720	0.0200		0.9%
L03	0.00075	0.00600	4	0.0195	0.03846	0.0200		-2.5%
L05	0.00300	0.02400	16	0.1101	0.02724	0.0799		27.4%
L05	0.00300	0.02400	16	0.1065	0.02817	0.0799		24.9%
L05	0.00300	0.02400	16	0.1104	0.02718	0.0799		27.6%
L06	0.00600	0.04800	32	0.1967	0.03050	0.1599		18.7%
L06	0.00600	0.04800	32	0.1983	0.03026	0.1599		19.4%
L06	0.00600	0.04800	32	0.1997	0.03005	0.1599		19.9%
L07	0.03000	0.24000	160	0.8076	0.03715	0.7993		1.0%
L07	0.03000	0.24000	160	0.8051	0.03726	0.7993		0.7%
L07	0.03000	0.24000	160	0.8088	0.03709	0.7993		1.2%
L08	0.06000	0.48000	320	1.5947	0.03763	1.5987		-0.3%
L08	0.06000	0.48000	320	1.5906	0.03772	1.5987		-0.5%
L08	0.06000	0.48000	320	1.5651	0.03834	1.5987		-2.1%
L09	0.15000	1.20000	800	3.4804	0.04310	3.9967		-14.8%
L09	0.15000	1.20000	800	3.4602	0.04335	3.9967		-15.5%
L09	0.15000	1.20000	800	3.4924	0.04295	3.9967		-14.4%
L10	0.30000	2.40000	1600	6.9093	0.04342	7.9935		-15.7%
L10	0.30000	2.40000	1600	7.0199	0.04274	7.9935		-13.9%
L10	0.30000	2.40000	1600	7.0593	0.04250	7.9935		-13.2%
L11	0.45000	3.60000	2400	10.5395	0.04270	11.9902		-13.8%
L11	0.45000	3.60000	2400	10.5063	0.04283	11.9902		-14.1%
L11	0.45000	3.60000	2400	10.4472	0.04307	11.9902		-14.8%
L12	0.60000	4.80000	3200	13.9292	0.04308	15.9870		-14.8%
L12	0.60000	4.80000	3200	13.9536	0.04300	15.9870		-14.6%
L12	0.60000	4.80000	3200	14.1050	0.04254	15.9870		-13.3%
L13	0.90000	7.20000	4800	21.6176	0.04163	23.9805		-10.9%
L13	0.90000	7.20000	4800	21.4033	0.04205	23.9805		-12.0%
L13	0.90000	7.20000	4800	20.6049	0.04368	23.9805		-16.4%
L14	1.08000	8.64000	5760	26.1828	0.04125	28.7766		-9.9%
L14	1.08000	8.64000	5760	25.7335	0.04197	28.7766		-11.8%
L14	1.08000	8.64000	5760	25.7859	0.04188	28.7766		-11.6%
L15	1.35000	10.80000	7200	33.3210	0.04052	35.9707		-8.0%
L15	1.35000	10.80000	7200	31.9497	0.04225	35.9707		-12.6%
L15	1.35000	10.80000	7200	31.6965	0.04259	35.9707		-13.5%

Acknowledgements: IROA-labeled precursors for the development of IS and LTRS were produced for IROA Technologies (under agreement) by CIL.

RESULTS

This experiment developed an efficient method to quantify multiple compounds in a single mixture.

1) The samples in the experimental design **(see Figure 1)** were analyzed according to two different workflows **(see Figure 2)**. Both are based on a single 96 well plate which contained 84 compounds at the same concentration.
2) **Workflow A** developed to generate a library of the exact chromatographic responses we expected to see for each compound analyzed in workflow B.
3) **Workflow B** used a pooled mixture of all 84 compounds to create a gradient of concentrated samples ("gradient injections" in triplicate). These gradient samples were dried under a gentle nitrogen stream, and 30 µL of a biological internal standard extract was added to resolvate the samples. Thus, each gradient sample contained a known concentration of each compound and a fixed amount of internal standard.
4) These resolvated gradient samples were injected (5 µL) following the library injections to keep solvent and column behavior consistent.
5) The expected concentration of each compound in the "gradient" samples is shown in **Figure 3**. We used a per injection concentration range of 0.00019 µg to 1.3500 µg (**Figure 3**), a **7200-fold increasing gradient** and a fixed concentration of IS. as shown in **Figure 4.**
6) For each compound in each sample the apical ratio of the natural abundance (summed) peaks to the ^{13}C isotopic (summed) peaks was calculated (**Figure 5C**). The concentration/the ratio for the samples most closely approximating 1:1 provided a measure of the amount of each compound in the internal standard. In **Figure 5** the 1:1 ratio was determined to be 0.0375 ug per injection. (highlighted in yellow)
7) The error in the ratio was then back calculated for each injection (**Figure 5**, white and yellow columns). Within three orders of magnitude down from the 1:1 ratio the error was approximately 20% or better, and increasing concentrations from 1:1 were generally showed less than 15% error.

CONCLUSIONS

The resultant quantification of the IS makes it possible to measure hundreds of metabolites more accurately than simple relative peak comparison. This will not be as accurate as absolute quantitation of each individual compound, but it is a step forward and within the error of any measurement in a semi-targeted metabolomics analysis.

REFERENCES

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