SUPPORTING INFORMATION FOR PUBLICATION

An improved method for measuring absolute metabolite concentrations in small biofluid or tissue samples

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S-1 Derivation of paired VDTS equations

The following computes the equations to be applied for the calculation of the derived parameters in the paired VDTS (pVDTS) protocol. In general, the symbols retain the definitions given in the Supporting Information to reference[1] which describes the original VDTS method. The following definitions apply:

V_h, volume of recovered hemolymph from a set of larvae;

V_t, volume transferred from the droplet for each of the two further 'unopened' and 'opened' polar metabolite extractions;

n_0^f, number of moles of sodium ^{13}C-formate employed in starting droplet, comprising a droplet volume V_f with concentration $[f]_0$;

n_0^D, number of moles of DSS employed in metabolite extraction procedure, comprising a volume V_D with concentration $[D]_0$;

I_i^f, signal intensity of the ^{1}H NMR signal for ^{13}C-formate in (diluted) NMR sample *i*, where *i* = 1 ('unopened'), or 2 (opened), expressed in concentration units and obtained by fitting the ^{1}H signals to those from a known concentration of sodium ^{13}C-formate standard;

I_i^D, signal intensity of the ^{1}H NMR for DSS in NMR sample *i*, expressed in concentration units and obtained by fitting the methyl group ^{1}H signal to that from a known concentration of DSS standard;

$I_i'^f$, concentration of ^{13}C-formate in NMR sample *i*, expressed in units of the DSS concentration, namely:

$$I_i'^f = I_i^f / I_i^D$$

The following relate to the initial conditions:

No. moles DSS used in extractions:

$$n_0^D = [D]_0 \cdot V_D$$

No. moles of formate standard *f* in droplet at start of experiment:

$$n_0^f = [f]_0 \cdot V_f$$

The concentration of formate standard in starting droplet is:

$$[f]_0 = \frac{n_0^f}{V_f}$$

The concentration of the formate standard after introducing larvae to the droplet for the 'control' (or 'unopened') NMR sample 1 (subscript '1'), where V_{co} is the carry-over 'wetness' volume from the flies is:

$$[f]_1 = \frac{n_0^f}{V_f} \cdot \left(\frac{V_f}{V_f + V_{co}}\right)$$

$$[f]_1 = \frac{n_0^f}{V_f + V_{co}}$$

No. moles of formate standard taken for first NMR measurement, in volume V_t:

$$n_1^f = V_t \cdot [f]_1 = \frac{V_t \cdot n_0^f}{V_f + V_{co}}$$

Assuming equal recovery efficiency for DSS and standard during the extraction, the ratio of Chenomx-interpreted concentrations of DSS:formate standard in the first 'control' or 'unopened' (subscript '1') NMR measurement is given by:

$$\left(\frac{I^D}{I^f}\right)_1 = \frac{n_0^D}{n_1^f} = \frac{n_0^D}{\left(\frac{V_t \cdot n_0^f}{V_f + V_{co}}\right)}$$

$$= \frac{n^D \cdot (V_f + V_{co})}{n_0^f \cdot V_t}$$

Rearranging for V_{co}:

$$n_0^D \cdot (V_f + V_{co}) = n_0^f \cdot V_t \cdot \left(\frac{I^D}{I^f}\right)_1$$

$$V_f + V_{co} = \frac{n_0^f \cdot V_t}{n_0^D} \cdot \left(\frac{I^D}{I^f}\right)_1$$

$$V_{co} = V_t \cdot \frac{n_0^f}{n_0^D} \cdot \left(\frac{I^D}{I^f}\right)_1 - V_f$$

Or:

$$V_{co} = V_t \cdot \Omega \cdot \frac{1}{I'^{f}_1} - V_f$$

Where:

$$\Omega = \frac{n^f_0}{n^D_0} = \frac{[f]_0 \cdot V_f}{[D]_0 \cdot V_D}$$

and the prime in I'^{f}_1 indicates that the concentration of formate is taken in units of the DSS level in the sample 1 as assessed by NMR, taking into account the number of protons in each signal (nine for the DSS trimethyl signal; 0.5 for each H-1 formate doublet component).

When a volume V_t is taken for the first ('unopened' or 'control') NMR sample, the number of moles of formate removed is:

$$n^f_1 = [f]_1 \cdot V_t$$

$$= \frac{n^f_0 \cdot V_t}{V_f + V_{co}}$$

The volume left in the droplet is: $V_f + V_{co} - V_t$

And the no. moles of formate remaining is:

$$n^f_0 - n^f_1 = n^f_0 - \frac{n^f_0 \cdot V_t}{V_f + V_{co}}$$

$$= n^f_0 \cdot \left(1 - \frac{V_t}{V_f + V_{co}}\right)$$

$$= n^f_0 \cdot \left(\frac{V_f + V_{co} - V_t}{V_f + V_{co}}\right)$$

When the larvae are opened the volume of the droplet now grows by V_h, so the concentration of formate is now:

$$[f]_2 = n^f_0 \cdot \frac{\left(\frac{V_f + V_{co} - V_t}{V_f + V_{co}}\right)}{V_f + V_{co} - V_t + V_h}$$

$$= n^f_0 \cdot \frac{V_f + V_{co} - V_t}{(V_f + V_{co})(V_f + V_{co} - V_t + V_h)}$$

The number of moles of formate taken for the 2nd NMR measurement is:

$$n_2^f = [f]_2 \cdot V_t = n_0^f \cdot \frac{V_t \cdot (V_f + V_{co} - V_t)}{(V_f + V_{co})(V_f + V_{co} - V_t + V_h)}$$

From the second, 'experimental' (or 'opened') NMR spectrum (subscript '2'), the ratio of *Chenomx Profiler*-interpreted concentrations of DSS:formate standard is given by:

$$\left(\frac{I^D}{I^f}\right)_2 = \frac{1}{I_2'^f} = \frac{n_0^D}{n_2^f} = \frac{n_0^D}{n_0^f \cdot \frac{V_t \cdot (V_f + V_{co} - V_t)}{(V_f + V_{co})(V_f + V_{co} - V_t + V_h)}}$$

$$= \left(\frac{n_0^D}{n_0^f}\right) \cdot \frac{(V_f + V_{co})(V_f + V_{co} - V_t + V_h)}{V_t \cdot (V_f + V_{co} - V_t)}$$

$$= \frac{1}{\Omega} \cdot \frac{(V_f + V_{co})(V_f + V_{co} - V_t + V_h)}{V_t \cdot (V_f + V_{co} - V_t)}$$

We know that: $V_{co} = V_t \cdot \Omega \cdot \frac{1}{I_1'^f} - V_f$

$$V_t \cdot \Omega \cdot \frac{1}{I_1'^f} - V_{co} = V_f$$

$$V_f + V_{co} = V_t \cdot \Omega \cdot \frac{1}{I_1'^f}$$

So: $\frac{1}{I_2'^f} = \frac{1}{\Omega} \cdot \frac{\left(V_t \cdot \Omega \cdot \frac{1}{I_1'^f}\right)\left(V_t \cdot \Omega \cdot \frac{1}{I_1'^f} - V_t + V_h\right)}{V_t \cdot \left(V_t \cdot \Omega \cdot \frac{1}{I_1'^f} - V_t\right)}$

$$\frac{1}{I_2'^f} = \frac{1}{I_1'^f} \cdot \frac{V_t \cdot \Omega \cdot \frac{1}{I_1'^f} - V_t + V_h}{V_t \cdot \Omega \cdot \frac{1}{I_1'^f} - V_t}$$

Solving for V_h:

$$\frac{\frac{1}{I_2'^f}}{\frac{1}{I_1'^f}} = \frac{I_1'^f}{I_2'^f} = \frac{V_t \cdot \Omega \cdot \frac{1}{I_1'^f} - V_t + V_h}{V_t \cdot \Omega \cdot \frac{1}{I_1'^f} - V_t}$$

$$V_t \cdot \Omega \cdot \frac{1}{I_1'^f} - V_t + V_h = \frac{I_1'^f}{I_2'^f} \cdot \left(V_t \cdot \Omega \cdot \frac{1}{I_1'^f} - V_t\right)$$

$$V_h = \frac{I_1'^f}{I_2'^f} \cdot \left(V_t \cdot \Omega \cdot \frac{1}{I_1'^f} - V_t \right) - \left(V_t \cdot \Omega \cdot \frac{1}{I_1'^f} - V_t \right)$$

$$V_h = V_t \cdot \left\{ \frac{I_1'^f}{I_2'^f} \cdot \left(\Omega \cdot \frac{1}{I_1'^f} - 1 \right) - \left(\Omega \cdot \frac{1}{I_1'^f} - 1 \right) \right\}$$

$$V_h = V_t \cdot \left(\Omega \cdot \frac{1}{I_1'^f} - 1 \right) \left(\frac{I_1'^f}{I_2'^f} - 1 \right)$$

$$V_h = V_t \cdot \left(\Omega \cdot \frac{1}{I_2'^f} - \frac{I_1'^f}{I_2'^f} - \Omega \cdot \frac{1}{I_1'^f} + 1 \right)$$

$$V_h = V_t \cdot \left\{ \Omega \cdot \left(\frac{1}{I_2'^f} - \frac{1}{I_1'^f} \right) - \frac{I_1'^f}{I_2'^f} + 1 \right\}$$

Now for the hemolymph metabolites:

Upon opening the flies for experiment '2' the physiologic concentration of hemolymph metabolite X $[X]_h$ is reduced in the droplet (prior volume $V_f + V_{co} - V_t$) to:

$$[X]_h \cdot \frac{V_h}{V_f + V_{co} + V_h - V_t}$$

And the number of moles of metabolite X removed for the second NMR sample is:

$$n^X = [X]_h \cdot \frac{V_h}{V_f + V_{co} + V_h - V_t} \cdot V_t$$

So in the second NMR experiment:

$$\left(\frac{I^X}{I^D} \right)_2 = \frac{n^X}{n_0^D} = \frac{[X]_h \cdot \frac{V_h}{V_f + V_{co} + V_h - V_t} \cdot V_t}{[D]_0 \cdot V_D}$$

Adopting

$$\left(\frac{I^X}{I^D} \right)_2 = I_2'^X$$

and rearranging yields:

$$[X]_h = I_2'^X \cdot \left(\frac{[D]_0 \cdot V_D}{V_t \cdot V_h} \right) (V_f + V_{co} + V_h - V_t)$$

Now, combining results from above:

$$V_{co} + V_h = V_t \cdot \Omega \cdot \frac{1}{I_1'^f} - V_f + V_t \cdot \left\{ \Omega \cdot \left(\frac{1}{I_2'^f} - \frac{1}{I_1'^f} \right) - \frac{I_1'^f}{I_2'^f} + 1 \right\}$$

$$= \; V_t \cdot \Omega \cdot \frac{1}{I_1'^f} - V_f + V_t \cdot \Omega \cdot \left(\frac{1}{I_2'^f} - \frac{1}{I_1'^f} \right) - V_t \cdot \frac{I_1'^f}{I_2'^f} + V_t$$

$$= V_t - V_f \; + V_t \cdot \Omega \cdot \frac{1}{I_2'^f} - V_t \cdot \frac{I_1'^f}{I_2'^f}$$

Substitute $V_{co} + V_h$ into:

$$[X]_h = \; I_2'^X \cdot \left(\frac{[D]_0 \cdot V_D}{V_t \cdot V_h} \right) (V_f + V_{co} + V_h - V_t)$$

$$[X]_h = \; I_2'^X \cdot \left(\frac{[D]_0 \cdot V_D}{V_t \cdot V_h} \right) \left(V_f + V_t - V_f \; + V_t \cdot \Omega \cdot \frac{1}{I_2'^f} - V_t \cdot \frac{I_1'^f}{I_2'^f} - V_t \right)$$

$$= \; I_2'^X \cdot \left(\frac{[D]_0 \cdot V_D}{V_t \cdot V_h} \right) \left(V_t \cdot \Omega \cdot \frac{1}{I_2'^f} - V_t \cdot \frac{I_1'^f}{I_2'^f} \right)$$

$$[X]_h = \; I_2'^X \cdot \left(\frac{[D]_0 \cdot V_D}{V_h} \right) \left(\Omega \cdot \frac{1}{I_2'^f} - \frac{I_1'^f}{I_2'^f} \right)$$

For practical calculations, we could stop here and use V_h formula from above. However, we can continue by substituting for V_h in denominator:

$$[X]_h = \; I_2'^X \cdot \left[\frac{[D]_0 \cdot V_D}{V_t \cdot \left\{ \Omega \cdot \left(\frac{1}{I_2'^f} - \frac{1}{I_1'^f} \right) - \frac{I_1'^f}{I_2'^f} + 1 \right\}} \right] \left(\Omega \cdot \frac{1}{I_2'^f} - \frac{I_1'^f}{I_2'^f} \right)$$

$$= \; I_2'^X \cdot [D]_0 \cdot V_D \cdot \left[\frac{\Omega \cdot \frac{1}{I_2'^f}}{V_t \cdot \left\{ \Omega \cdot \left(\frac{1}{I_2'^f} - \frac{1}{I_1'^f} \right) - \frac{I_1'^f}{I_2'^f} + 1 \right\}} - \frac{\frac{I_1'^f}{I_2'^f}}{V_t \cdot \left\{ \Omega \cdot \left(\frac{1}{I_2'^f} - \frac{1}{I_1'^f} \right) - \frac{I_1'^f}{I_2'^f} + 1 \right\}} \right]$$

$$= I_2'^X \cdot [D]_0 \cdot V_D \cdot \left[\frac{\frac{1}{I_2'^f}}{V_t \cdot \left\{ \left(\frac{1}{I_2'^f} - \frac{1}{I_1'^f} \right) - \frac{1}{\Omega} \cdot \frac{I_1'^f}{I_2'^f} + \frac{1}{\Omega} \right\}} - \frac{\frac{1}{I_2'^f}}{V_t \cdot \left\{ \Omega \cdot \frac{1}{I_1'^f} \cdot \left(\frac{1}{I_2'^f} - \frac{1}{I_1'^f} \right) - \frac{1}{I_2'^f} + \frac{1}{I_1'^f} \right\}} \right]$$

$$= I_2'^X \cdot [D]_0 \cdot \frac{V_D}{V_t} \cdot \frac{1}{I_2'^f} \cdot \left\{ \frac{1}{\left(\frac{1}{I_2'^f} - \frac{1}{I_1'^f} \right) - \frac{1}{\Omega} \cdot \frac{I_1'^f}{I_2'^f} + \frac{1}{\Omega}} - \frac{1}{\Omega \cdot \frac{1}{I_1'^f} \cdot \left(\frac{1}{I_2'^f} - \frac{1}{I_1'^f} \right) - \frac{1}{I_2'^f} + \frac{1}{I_1'^f}} \right\}$$

$$= I_2'^X \cdot [D]_0 \cdot \frac{V_D}{V_t} \cdot \frac{1}{I_2'^f} \cdot \left\{ \frac{\frac{1}{I_1'^f}}{\frac{1}{I_1'^f} \cdot \left(\frac{1}{I_2'^f} - \frac{1}{I_1'^f} \right) - \frac{1}{\Omega} \cdot \frac{1}{I_2'^f} + \frac{1}{\Omega} \cdot \frac{1}{I_1'^f}} - \frac{1}{\Omega \cdot \frac{1}{I_1'^f} \cdot \left(\frac{1}{I_2'^f} - \frac{1}{I_1'^f} \right) - \frac{1}{I_2'^f} + \frac{1}{I_1'^f}} \right\}$$

$$= I_2'^X \cdot [D]_0 \cdot \frac{V_D}{V_t} \cdot \frac{1}{I_2'^f} \cdot \left\{ \frac{\Omega \cdot \frac{1}{I_1'^f}}{\Omega \cdot \frac{1}{I_1'^f} \cdot \left(\frac{1}{I_2'^f} - \frac{1}{I_1'^f} \right) - \frac{1}{I_2'^f} + \frac{1}{I_1'^f}} - \frac{1}{\Omega \cdot \frac{1}{I_1'^f} \cdot \left(\frac{1}{I_2'^f} - \frac{1}{I_1'^f} \right) - \frac{1}{I_2'^f} + \frac{1}{I_1'^f}} \right\}$$

$$= I_2'^X \cdot [D]_0 \cdot \frac{V_D}{V_t} \cdot \frac{1}{I_2'^f} \cdot \left\{ \frac{\Omega \cdot \frac{1}{I_1'^f} - 1}{\Omega \cdot \frac{1}{I_1'^f} \cdot \left(\frac{1}{I_2'^f} - \frac{1}{I_1'^f} \right) - \frac{1}{I_2'^f} + \frac{1}{I_1'^f}} \right\}$$

$$= I_2'^X \cdot [D]_0 \cdot \frac{V_D}{V_t} \cdot \frac{1}{I_2'^f} \cdot \left\{ \frac{\Omega \cdot \frac{1}{I_1'^f} - 1}{\left(\Omega \cdot \frac{1}{I_1'^f} - 1 \right) \cdot \left(\frac{1}{I_2'^f} - \frac{1}{I_1'^f} \right)} \right\}$$

$$= I_2'^X \cdot [D]_0 \cdot \frac{V_D}{V_t} \cdot \frac{1}{I_2'^f} \cdot \left\{ \frac{1}{\left(\frac{1}{I_2'^f} - \frac{1}{I_1'^f} \right)} \right\}$$

$$[X]_h = I_2'^X \cdot \frac{[D]_0 \cdot V_D}{V_t} \cdot \left\{ \frac{\frac{1}{I_2'^f}}{\left(\frac{1}{I_2'^f} - \frac{1}{I_1'^f} \right)} \right\}$$

$$[X]_h = I_2'^X \cdot \frac{[D]_0 \cdot V_D}{V_t} \cdot \left\{ \frac{1}{\left(1 - \frac{I_2'^f}{I_1'^f} \right)} \right\}$$

$$[X]_h = I_2'^X \cdot [D]_0 \cdot \frac{V_D}{V_t} \cdot \left(1 - \frac{I_2'^f}{I_1'^f} \right)^{-1}$$

S-2 Consideration of potential sources of error

There are three potential sources of error that we have considered:

1) The assumed concentration of sodium ^{13}C-formate in the starting droplet. The observed concentration in the NMR spectrum, assessed by comparison to the DSS standard, could be in error and thereby affect the value input to the *Chenomx NMR Suite* software for the reference spectrum of the sodium ^{13}C-formate. This error can be circumvented by assessing the relative integrals of the DSS trimethyl signal (δ = 0 ppm) versus the 1-H signals for ^{13}C-HCO_2^- in a 'fully relaxed' standard pulse-and-acquire 1D NMR spectrum recorded with long relaxation delay (greater than 5 * longest T_1 relaxation time constant; in our hands 45 s). Taking this precaution can be equated to introducing the correction factor *x* in the following normalizations:

$$I_i'^f = \left(x \cdot \frac{I^f}{I^D} \right)_i$$

2) Conceptually, the result V_{co} = 0 should be obtained if a separate control experiment is performed similar to the strand '1' ('unopened') experiment described in the pVDTS procedure (see main text) but where no larvae are introduced. For a batch of such 'straight-though' control replicates, we find that the V_{co} values obtained can cluster around a non-zero value. Given that:

$$V_{co} = \frac{V_t}{\Omega} \cdot \left(\frac{I^D}{I^f} \right)_1 - V_f \quad ; \quad \Omega = \frac{n_0^D}{n_0^f} = \frac{[D]_0 \cdot V_D}{[f]_0 \cdot V_f}$$

and assuming that V_f and V_t are similarly measured to high precision using a Hamilton syringe, then a non-zero V_{co} for the straight-through controls implies an error in the assumed value of Ω versus that based on the gravimetric dispensing of sodium ^{13}C-formate and DSS standards, and the volumetric dilutions of the starting (high concentration) stock solutions applied to obtain the final experimental reagents. For each batch of samples used in a pVDTS experiment, we recommend that a number of 'straight-through' control experiments are performed to assess the magnitude of any such discrepancy. A numerical correction (here denoted y) for this discrepancy can be applied in calculations of V_{co} and V_h:

$$V_{co} = \frac{y \cdot V_t}{\Omega} \cdot \left(\frac{x \cdot I^D}{I^f} \right)_1 - V_f$$

$$V_h = V_t \cdot \left[\frac{y.x}{\Omega} \cdot \left\{ \left(\frac{I^D}{I^f} \right)_2 - \left(\frac{I^D}{I^f} \right)_1 \right\} - \frac{\left(\frac{I^D}{I^f} \right)_2}{\left(\frac{I^D}{I^f} \right)_1} + 1 \right]$$

3) It is noteworthy that since neither V_{co} nor V_h appear directly in the equation for the target metabolite absolute concentrations $[X]_h$ and all instances of I'^f normalize out the need for x (see derivation above), any final errors are collected in the quotient:

$$[D]_0 \cdot \frac{V_D}{V_t}$$

Consistent pipetting (for dispensing volumes V_t and V_D) and the use of a 'gold standard' stock solution of DSS in the preparation (by dilution from that 'gold standard' stock) of the DSS solution at concentration $[D]_0$ used in the experiments will minimize any variation of the error in $[X]_h$ values obtained across different studies.

A comment on the precision of the measurement:

The 'errors' (uncertainty) in the quantities derived in the pVDTS workflow reflect both biological and technical variability. The precision can be estimated as follows: based on the concepts of error propagation an individual *n* % error in either of the pipetted volumes V_t and V_D would each rise to a proportionate error of *n* % in the derived values for metabolite concentrations $[X]_h$. If the errors in pipetting are in the same direction (both high, or both low), then these errors would tend to reduce the overall error in $[X]_h$. If the errors are in the opposite direction, then they would combine to yield a larger error. We anticipate that pipetting errors would be relatively small: < 2.5%. Note that any pipetting error would apply to any given instance of the NMR measurement and therefore there would be no errors in

the *comparative* values of $[X]_h$. A second potential source of imprecision derives from the bracketed term in Equation 6 (see main text), namely:

$$\left(1-\frac{I_2'^f}{I_1'^f}\right)^{-1}$$

that can be rewritten:

$$\frac{I_1'^f}{I_1'^f - I_2'^f}$$

This term contains the difference $I_1'^f - I_2'^f$ which will be increasing susceptible to error with smaller volume of released hemolymph. Modelling of the derived error in $[X]_h$ for a 1% and 2% error in the measurement of $I_1'^f$ and $I_2'^f$ yields the following inaccuracies for $[X]_h$:

	% Error in $[X]_h$				
$I_2'^f/I_1'^f$	+1 % error in $I_1'^f$	+1 % error in $I_2'^f$	+1 % error in both $I_1'^f$ and $I_2'^f$	+1 % error in $I_1'^f$ / -1 % error in $I_2'^f$	-1 % error in $I_1'^f$ / +1 % error in $I_2'^f$
0.9	-8	10	0	-15	22
0.8	-4	4	0	-7	9
0.7	-2	2	0	-4	5
0.6	-1	2	0	-3	3
0.5	-1	1	0	-2	2
0.4	-1	1	0	-1	1
0.3	0	0	0	-1	1
0.2	0	0	0	0	1
0.1	0	0	0	0	0

	% Error in $[X]_h$				
$I_1'^f/I_2'^f$	+2 % error in $I_1'^f$	+2 % error in $I_2'^f$	+2 % error in both $I_1'^f$ and $I_2'^f$	+2 % error in $I_1'^f$ / -2 % error in $I_2'^f$	-2 % error in $I_1'^f$ / +2 % error in $I_2'^f$
0.9	-15	22	0	-26	58
0.8	-7	9	0	-14	20
0.7	-4	5	0	-8	11
0.6	-3	3	0	-6	7

0.5	-2	2	0	-4	4
0.4	-1	1	0	-3	3
0.3	-1	1	0	-2	2
0.2	0	1	0	-1	1
0.1	0	0	0	0	0

These results indicate that error in the derived value of $[X]_h$ is minimized: (a) for higher values of the dilution of the ^{13}C-formate NMR standard in the initial droplet; (b) for metabolites with more intense NMR resonances (when the fitting error in estimating $I_1'^f$ and $I_2'^f$ will be smaller); and (c) when the error in the latter quantities is in the same direction (both positive, or both negative) as these tend to be self-compensating for any value of ratio $I_2'^f/I_1'^f$. With respect to (a) it is recommended that the target volume of pVDTS represents the highest fraction of the starting droplet volume (V_f) that is achievable (herein ~0.3). With respect to (c), we note that any error in obtaining the fit of the NMR resonances of the target metabolite X to the corresponding library spectrum is likely to have the same sign as that in fitting the NMR standards ^{13}C-formate and DSS due to any uncertainty in the location of the spectrum baseline in areas of multiple peak overlap.

S-3 Mock hemolymph release experiments using pVDTS and VDTS workflows

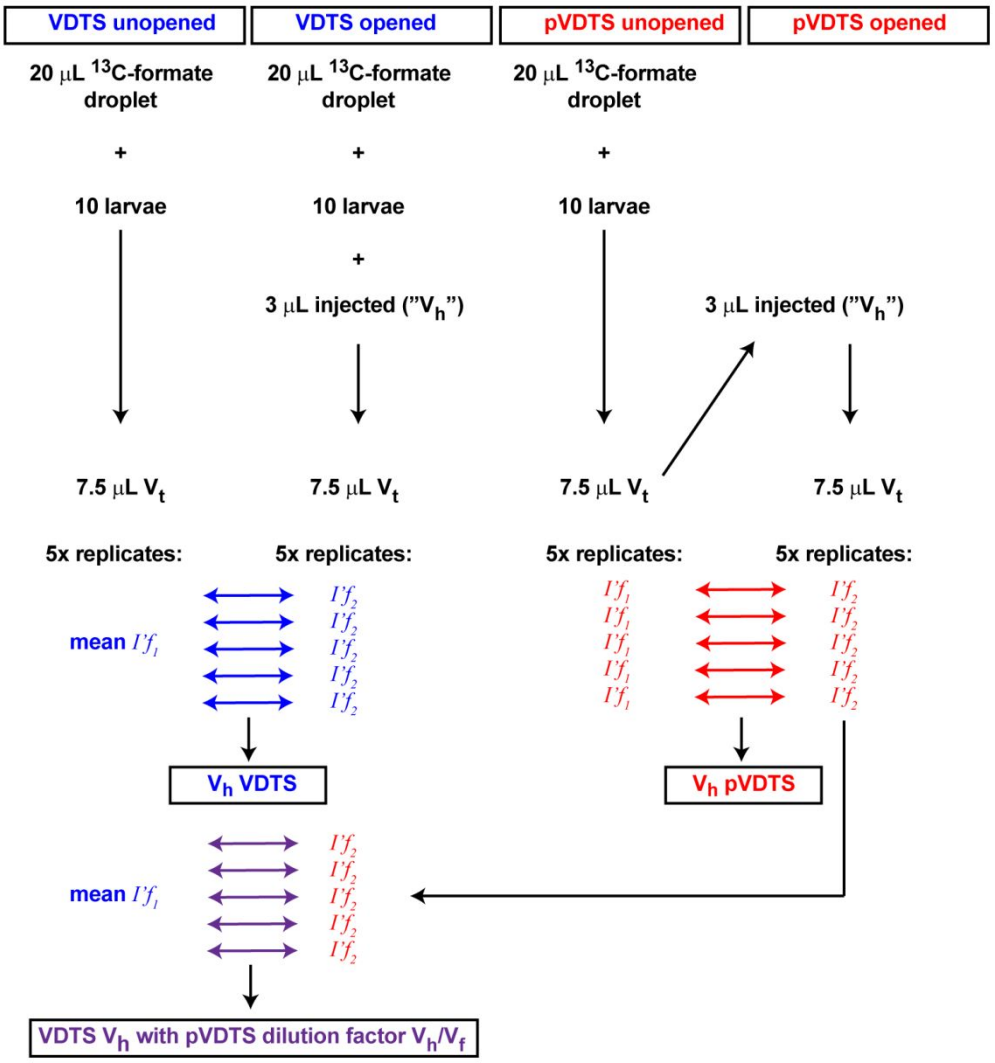
	Experimental outcome (a)			
Workflow	**I'^f_1 / unopened (CV)**	**I'^f_2 / opened (CV)**	**V_h (µL)**	**[metabolite] *Sc*Inositol:fumarate:NAD$^+$**
pVDTS	2.03 ± 0.04 (2.1%)	1.58 ± 0.04 (2.3%)	3.11 ± 0.43 (14%)	0.99:0.96:1
VDTS	2.02 ± 0.02 (0.9%)	1.82 ± 0.09 (4.7%)	2.28 ± 1.06 (47%)	0.97:0.93:1
pVDTS with VDTS measures of I'^f_1 (b)			3.50 ± 0.38 (11%)	0.97:0.93:1
Straight injection				1:1:1

Notes:

(a) See schematic below for experiment design.

(b) Combination the 'unopened' NMR measurements of the VDTS arm of the comparative mock experiment with the 'opened' NMR measurements of the pVDTS arm, demonstrating that it is likely that the smaller volume in which the larvae are opened in the pVDTS workflow, compared to VDTS, contributes to its superior precision (see also S-5). Worthy of note here is the relatively tight clustering of the I'^f_1 values for the

VDTS experiment, indicating very low dispersion of any carry over volumes V_{co} in this experiment.



S-4 *In silico* modelling of V_{co} and droplet size effects on the estimation of V_h

Simulations were performed for the following circumstances:

i) pVDTS using a droplet size of 20 µL;
ii) VDTS using a droplet size of 20 µL;
iii) VDTS using a droplet size of 12.5 µL, which corresponds to the situation for 'sample 2' (opened) of the pVDTS workflow, wherein the released hemolymph is released into a droplet from which the 'sample 1' (unopened) V_t aliquot (7.5 µL) has already been removed.

For the simulations the released volume of hemolymph V_h was assumed to be 3 µL. Random noise on the pipetted volumes V_f, V_t and V_D, representing technical variation on the part of the experimenter, was simulated at the level of ±0.1 µL (for V_f and V_t, Hamilton syringe) and ±1 µL for V_D (micropipettor), using the *Rand()* function in Microsoft Excel. Simulations were preformed assuming (a) nil V_{co}; (b); a random value for V_{co} in the range 0-1 µL; (c) as (b) but setting one value (of five experiments) of V_{co} to 5 µL, corresponding to a 'rogue', significantly wetter batch of larvae; and (d) a random value for V_{co} in the range 0-5 µL, corresponding to a series of potentially wetter larvae. The following table reports the typical values of the recovered value for the mean value of V_h obtained in such simulations:

	Simulated V_h (µL) estimation (with coefficient of variation)		
V_{co}	**pVDTS V_f = 20 µL**	**VDTS V_f = 20 µL**	**VDTS V_f = 12.5 µL**
0 µL	3.00 ± 0.04 (1.5%)	2.90 ± 0.19 (6.4%)	2.95± 0.17 (5.8%)
<0-1 µL>	2.99 ± 0.05 (1.6%)	3.17 ± 0.29 (9.0%)	3.21 ± 0.31 (9.8%)
<0-1 µL> plus once 5 µL in 5 experiments	3.01 ± 0.05 (1.7%)	3.05 ± 0.31 (10.3%)	3.27 ± 0.41 (12.5%)
<0-5 µL>	3.01 ± 0.05 (1.7%)	2.93 ± 1.71 (59%)	3.36 ± 1.25 (37%)

The results of the simulations indicate that both the droplet size V_f and the values of carry over volume V_{co} have direct impact upon the precision of the apparent value for V_h that is obtained in the VDTS workflow. pVDTS always yields a more precise V_h estimate, irrespective of the pattern of V_{co} volumes encountered.

S-5: Hemolymph metabolite concentrations calculated using the data averaging formulation from the VDTS method

Hemolymph concentrations determined for fed larvae from the spectra obtained using the paired VDTS workflow but estimated using the averaging formulation adopted in the original VDTS method [1]. Entries show mean concentration ±1 standard deviation for three independent experiments, each with at least three biological replicates for each sex.

	concentration (mM)		
Metabolite	Male	Female	p-value
alanine	8.23 ± 6.88	4.03 ± 1.95	0.58
arginine	1.34 ± 1.27	1.12 ± 0.48	0.98
aspargine	4.93 ± 3.79	3.63 ± 1.77	0.98
betaine	0.35 ± 0.26	0.47 ± 0.31	0.98
dimethylamine	0.06 ± 0.04	0.05 ± 0.02	0.98
fumarate	0.29 ± 0.21	0.22 ± 0.09	0.98
glucose	1.05 ± 1.22	0.94 ± 0.69	0.98
glutamine	19.78 ± 14.01	16.37 ± 7.80	0.98
glycine	2.58 ± 1.98	1.59 ± 0.73	0.85
histidine	3.70 ± 2.72	2.96 ± 1.54	0.98
isoleucine	0.65 ± 0.43	0.31 ± 0.11	0.18
leucine	1.17 ± 0.78	0.58 ± 0.20	0.24
lysine	5.93 ± 3.95	4.02 ± 1.80	0.90
malate	4.48 ± 3.27	3.39 ± 1.78	0.98
methionine	0.68 ± 0.45	0.54 ± 0.20	0.98
O-phosphocholine	3.14 ± 1.79	2.05 ± 0.76	0.65
O-phosphotyrosine	92.35 ± 60.04	72.24 ± 29.63	0.98
phenylalanine	0.41 ± 0.25	0.26 ± 0.09	0.57
proline	12.29 ± 7.35	8.94 ± 3.96	0.94
sarcosine	0.50 ± 0.33	0.24 ± 0.11	0.21
succinate	1.39 ± 0.76	1.05 ± 0.40	0.94
taurine	1.36 ± 0.85	1.14 ± 0.62	0.98
threonine	6.73 ± 4.97	4.86 ± 2.38	0.97
trehalose	103.10 ± 68.90	76.47 ± 31.04	0.97
trypothphan	0.30 ± 0.17	0.24 ± 0.12	0.98
tyrosine	7.29 ± 4.87	4.38 ± 2.03	0.65
valine	2.68 ± 1.81	1.39 ± 0.72	0.39
β-alanine	2.07 ± 1.40	1.72 ± 0.79	0.98

References

1. Ragan, T. J.; Bailey, A. P.; Gould, A. P.; Driscoll, P. C., Volume Determination with Two Standards Allows Absolute Quantification and Improved Chemometric Analysis of Metabolites by NMR from Submicroliter Samples. *Anal Chem* **2013,** *85* (24), 12046-12054.