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Volume Determination with Two Standards Allows Absolute Quantification and Improved Chemometric Analysis of Metabolites by NMR from Submicroliter Samples

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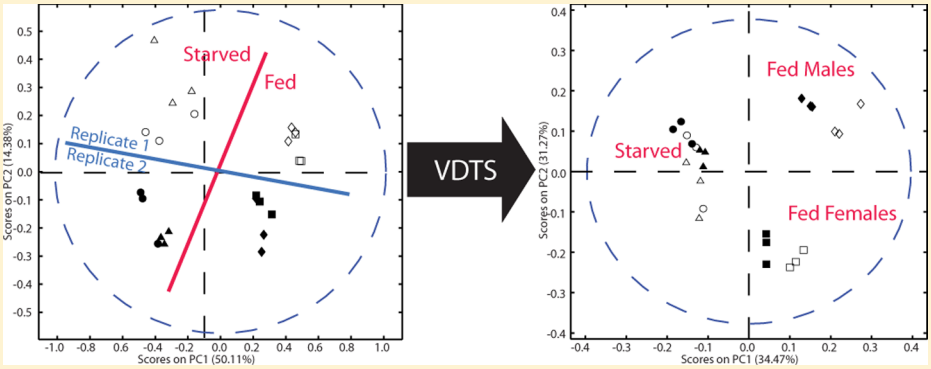
Supporting Information



ABSTRACT: The accurate measurement of metabolite concentrations in miniscule biological sample volumes is often desirable, yet it remains challenging. In many cases, the starting analyte volumes are imprecisely known, or not directly measurable, and hence absolute metabolite concentrations are difficult to calculate. Here, we introduce volume determination using two standards (VDTS) as a general quantitative method for the analysis of polar metabolites in submicrolitre samples using ^{1}H NMR spectroscopy. This approach permits the back calculation of absolute metabolite concentrations from small biological samples of unknown volume. Where small sample volumes are also variable, VDTS can improve multivariate chemometric analysis. In this context, principal component analysis (PCA) yielded more logically consistent and biologically insightful outputs when we used volume-corrected spectra, calculated using VDTS, rather than probabilistic quotient normalization (PQN) of raw spectra. As proof-of-principle, the VDTS-based method and PCA were used to analyze polar metabolites in the hemolymph (blood) extracted from larvae of the very small but widely used genetic model organism *Drosophila*. This analysis showed that the hemolymph metabolomes of males and females are markedly different when larvae are well fed. However, gender-specific metabolomes tend to converge when larval dietary nutrients are restricted. We discuss the biological implications of these surprising results and compare and contrast them to previous analyses of *Drosophila* hemolymph and mammalian blood plasma. Together, these findings reveal an interesting and hitherto unknown sexual dimorphism in systemic *Drosophila* metabolites, clearly warranting further biological investigation. Importantly, the VDTS approach should be adaptable to many different analytical platforms, including mass spectrometry.

Metabolomic profiling, either by mass spectrometry (MS) or nuclear magnetic resonance (NMR) spectroscopy, is increasingly being used to provide new insights into fundamental biological processes in a multitude of different species. With known sample volumes of relatively abundant material, the absolute quantification of metabolite concentration is, in principle, straightforward by measurement of peak intensities after normalization for environmental variation or instrumental instability. For example, in many applications of metabolomics to human body fluids such as urine, plasma or cerebrospinal fluid (CSF), the volume of the starting sample can be accurately measured. Thus the absolute concentration of each metabolite is accessible by reference to a single internal standard and by accounting for dilution during sample preparation. However, many tissue biopsies and rarer human fluids such as endolymph are only available in limiting and variable amounts of unknown sample volume. Such "difficult" small samples not only have the potential to compromise measurement sensitivity, but their variability challenges many data normalization methods. Where

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imprecisely known small samples *require* dilution to provide a sample of sufficient volume for the analytical measurement, the extent to which metabolites are diluted (the sample-specific 'dilution factor') is either not known or inaccurately determined. As a result, although measurement of the metabolite concentrations in the *diluted* sample is straightforward, back-calculation of the absolute concentrations in the starting material is inherently compromised. To avoid these difficulties, many studies resort to the measurement of relative changes, rather than absolute concentrations of the metabolites, using the methods of chemometric multivariate analysis. Here issues related to absolute metabolite concentration are taken care of by "statistical normalization". In many instances the method of choice is probabilistic quotient normalization (PQN),[1] which outperforms a number of other means of normalization (e.g., integral normalization and vector length normalization). PQN accounts for specific and unspecific variation in a set of measurements, corresponding to changes in a minority subset of spectral features and the total spectral intensity, respectively. However its successful application is limited by the requirement that relative concentrations of the profiled metabolites (or more strictly the intensities of the corresponding spectral features) do not vary by more than ∼50%.[1]

The fruitfly *Drosophila melanogaster* has become an increasingly powerful genetic model for the study of metabolism during growth, development and aging.[2−4] However, the very small size of the organism has been a hindrance for the accurate measurement of metabolite concentrations in individual tissues or within the circulating body fluid, known as hemolymph. As part of ongoing investigations into the roles of dietary nutrients during the growth of *Drosophila*, we have monitored changes in polar hemolymph metabolites upon nutrient restriction (NR) during larval development.[5] The inherent small size of the organism, the fully grown larva is ∼1 × 0.5 mm (length × width), equates to hemolymph sample volumes that are of the order of only a few hundred nanoliters. This, together with their high viscosity, makes it difficult to measure hemolymph volumes accurately using a direct method. Moreover, in comparing different strains of the organism, or the response of a single strain to different growth conditions, it is difficult to assess whether or not the recovered hemolymph volumes might vary in any systematic way.

^{1}H NMR spectroscopy can be used to provide a relatively unbiased approach to metabolomics investigation.[6−17] Here, we describe the development of methods for the application ^{1}H NMR to the analysis of polar metabolites in small and variable samples of unknown starting volume. We select *Drosophila* larval hemolymph as a challenging test case and introduce the method of volume determination using two standards (VDTS) to assess accurately the sample dilution and original analyte volume. We report the concentrations of the most abundant polar hemolymph metabolites and provide a comparative analysis of the hemolymph metabolome under fed and nutrient restriction (NR) conditions in both male and female larvae. Application of VDTS combined with supervised fitting of the NMR spectra obtained for four different conditions (male vs female, fed vs NR) allows a quantitative assessment of each metabolite concentration, revealing previously unreported gender-specific variation of the hemolymph metabolome under fed conditions that is suppressed under NR. Moreover, it is demonstrated that standard application of PQN analysis to the raw data without correction for the sample-to-sample dilution factor fails to provide a physically self-consistent set of peak intensities in the loadings plot. However, by using VDTS to facilitate normalizing the data set with respect to absolute concentration, the PCA output is rendered sensible. This approach allows for the facile determination of the sample-to-sample variance and, via analysis of the corresponding loadings plots, the identification of molecular species contributing to that variance. The principle of VDTS is thus demonstrated to be a valuable tool in metabolomics analysis of small and variable sample volumes, and is likely to be of general utility in measurement paradigms other than NMR spectroscopy.

EXPERIMENTAL SECTION

Larvae of a *Drosophila melanogaster* isogenic strain (w1118 iso 31)[18] were transferred to standard yeast/cornmeal/agar food within 1 h of hatching and raised at 25 °C for 68 h until mid-L3 (larval wet weight ∼1.3 mg). Larvae were then floated from the food with 30% glycerol/phosphate-buffered saline (PBS) and transferred to control diet (standard yeast/cornmeal/agar food) or a nutrient restriction medium (0.5% agarose in PBS) for a further 20 h at 25 °C.

For hemolymph isolation in batch mode, larvae were floated in 30% glycerol/PBS and rinsed in water, and groups of 10 (fed condition) or 15 (NR condition) larvae were blotted dry on tissue paper and placed on Parafilm. Using a Hamilton syringe to ensure accuracy, a 20 μL drop of ice cold saline with 100 μM sodium ^{13}C-formate was placed on the group of larvae, and sharp forceps were used to tear the cuticle of each larva, releasing the hemolymph into the droplet, but without damaging any internal tissues. All larvae were opened within 2 min, and 17.5 μL of the saline/sodium formate/hemolymph solution was then removed with the Hamilton syringe and added to 200 μL reverse-osmosis deionized water containing 80 μM sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), and any cells, such as hemocytes, present in the hemolymph were removed by centrifugation (2 min at 13 krpm, Millipore UFC30GV0S 0.22 μm filter). To control for nonspecific interaction of the sodium formate with the larval cuticle, another group of larvae were treated as described but were not opened in the droplet and, therefore, did not release hemolymph. To control for evaporation of the drop during the sampling procedure, a 20 μL drop (no larvae) was placed on the Parafilm, left for 2 min, and 17.5 μL was removed and treated as described.

A similar procedure was used to prepare hemolymph extracts starting from individual larvae: the volume of the saline/sodium ^{13}C-formate drop was reduced to 3 μL, and after opening the larva, 2.5 μL was removed and added to 200 μL water containing 8 μM DSS before centrifugation.

Methanol−chloroform metabolite extraction was performed using the method of Bligh and Dyer.[19] Briefly, the aqueous sample was mixed with 750 μL 2:1 methanol-chloroform by vortexing. Phase separation was accomplished by the addition of 250 μL chloroform followed by 250 μL H_2O. The upper (aqueous) layer was evaporated to dryness (Savant DNA 120 centrifugal evaporator), resuspended in 160 μL 99.9% D_2O and transferred to a 3 mm NMR tube (Bruker Biospin).

NMR spectra were acquired with a Bruker Avance II instrument with a nominal ^{1}H frequency of 700 MHz using a CPTCI cryoprobe. The Bruker pulse program *noesygppr1d* was used with a 1 s presaturation pulse (50 Hz bandwidth) centered on the water resonance, 0.1 ms mixing time, and 4 s acquisition time at 25 °C. For chemometric analysis the data sets were zero-filled, Fourier-transformed and phase corrected in *NMRLab*.[20] Spectra were then transferred to *Metabolab*,[20] and baseline

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Table 1. Absolute Metabolite Concentrations in Fed and Nutrient-Restricted *Drosophila* Larvae Obtained by NMR Spectroscopy and VDTS[a]

	fed concentration (mM)[b]			NR concentration (mM)[b]		
metabolite	male	female	*p*-value[c]	male	female	*p*-value[c]
			α-amino acids			
alanine	**5.18 ± 1.00**	**3.48 ± 1.14**	**2.93×10^{-3}**	2.64 ± 0.76	2.32 ± 0.37	0.528
arginine	**0.879 ± 0.218**	**0.480 ± 0.010**	**6.41×10^{-3}**	0.555 ± 0.283	0.638 ± 0.263	0.534
asparagine	**2.25 ± 0.37**	**1.72 ± 0.20**	**1.95×10^{-3}**	0.725 ± 0.084	0.925 ± 0.176	0.193
aspartate[d]	<0.02	<0.02		<0.01	<0.01	
cysteine[d]	<0.1	<0.05		<0.02	<0.03	
glutamine	10.0 ± 1.9	9.18 ± 0.97	0.312	3.07 ± 0.72	4.32 ± 1.63	0.135
glutamate[d]	<0.2	<0.2		<0.12	<0.04	
glycine	**2.01 ± 0.20**	**1.62 ± 0.18**	**7.8×10^{-4}**	0.608 ± 0.100	0.828 ± 0.185	3.52×10^{-2}
histidine	1.53 ± 0.18	1.16 ± 0.15	0.156	0.929 ± 0.461	1.37 ± 0.71	9.49×10^{-2}
isoleucine	**0.281 ± 0.036**	**0.139 ± 0.025**	**1.03×10^{-4}**	0.148 ± 0.012	0.150 ± 0.018	0.752
leucine	**0.530 ± 0.055**	**0.265 ± 0.463**	**1×10^{-4}**	0.236 ± 0.026	0.257 ± 0.042	0.410
lysine	**4.25 ± 0.78**	**3.06 ± 0.45**	**7.4×10^{-4}**	1.04 ± 0.21	1.24 ± 0.44	0.492
methionine	**0.294 ± 0.044**	**0.184 ± 0.044**	**1.1×10^{-4}**	0.128 ± 0.036	0.112 ± 0.024	0.404
phenylalanine	**0.182 ± 0.027**	**0.0939 ± 0.0115**	**1×10^{-4}**	0.0486 ± 0.0063	0.0495 ± 0.0076	0.926
proline	**7.20 ± 1.85**	**5.17 ± 0.64**	**2.53×10^{-3}**	1.30 ± 0.40	1.54 ± 0.34	0.683
serine[d]	<0.5	<0.4		<0.5	<0.5	
threonine	**3.86 ± 0.53**	**3.36 ± 0.32**	**1.2×10^{-2}**	0.260 ± 0.044	0.371 ± 0.098	0.545
tryptophan	0.196 ± 0.040	0.163 ± 0.011	6.71×10^{-2}	0.0761 ± 0.0339	0.0798 ± 0.0219	0.829
tyrosine	**3.68 ± 0.57**	**2.22 ± 0.37**	**1×10^{-4}**	0.943 ± 0.117	0.874 ± 0.119	0.735
valine	**1.15 ± 0.20**	**0.656 ± 0.128**	**1×10^{-4}**	0.259 ± 0.039	0.262 ± 0.053	0.961
			other amino acids			
betaine	0.0963 ± 0.0244	0.0549 ± 0.0189	0.475	0.103 ± 0.084	0.233 ± 0.176[e]	3.37×10^{-2}
β-alanine	0.855 ± 0.284	0.712 ± 0.188	0.167	0.218 ± 0.022	0.269 ± 0.051	0.619
ornithine[d]	<0.6	<0.15		<0.2	<0.3	
sarcosine	1.06 ± 0.30	1.23 ± 0.19	0.118	**1.05 ± 0.042**	**1.50 ± 0.09**	**5.3×10^{-4}**
o-phosphotyrosine	**69.3 ± 8.8**	**48.1 ± 5.8**	**1.03×10^{-4}**	18.8 ± 1.4	17.8 ± 1.1	0.752
			TCA cycle			
fumarate	**0.243 ± 0.026**	**0.179 ± 0.043**	**1.65×10^{-2}**	0.163 ± 0.055	0.175 ± 0.041	0.617
malate	1.63 ± 0.40	1.32 ± 0.31	0.178	1.16 ± 0.47	1.35 ± 0.31	0.411
succinate	**0.501 ± 0.091**	**0.392 ± 0.030**	**1.81×10^{-2}**	**0.265 ± 0.039**	**0.378 ± 0.105**	**1.55×10^{-2}**
			carbohydrates			
glucose	0.334 ± 0.212	0.222 ± 0.107	0.141	0.131 ± 0.794	0.222 ± 0.034	0.228
trehalose	**53.8 ± 16.1**	**37.8 ± 9.4**	**1.38×10^{-3}**	30.2 ± 4.8	38.1 ± 6.8	0.194
			others			
carnitine[d]	<0.001	<0.001		<0.001	<0.001	
dimethylamine[f]	0.189 ± 0.043	0.0638 ± 0.0072	0.732	0.630 ± 0.720	0.742 ± 1.01	0.756
2-hydroxyglutarate[g]	1.02 ± 0.52	0.529 ± 0.298	2.69×10^{-2}	0.426 ± 0.366	0.168 ± 0.054	0.220
phosphocholine	**1.35 ± 0.27**	**0.966 ± 0.147**	**3.09×10^{-3}**	**1.85 ± 0.15[e]**	**2.22 ± 0.20[e]**	**4.29×10^{-3}**
taurine	0.737 ± 0.133	0.592 ± 0.084	0.249	0.743 ± 0.267	0.740 ± 0.292	0.977

[a]Bold figures indicate statistically significant differences *between males and females* for either the fed or nutrient-restricted (NR) conditions. [b]Entries are "concentration ± standard deviation" for two independent experiments each performed for three sets of fed or NR larvae, specified to three significant digits. [c]Statistical test of the difference in metabolite levels between male and female larvae; bold text indicates a significant difference according to Tukey's honest significant difference (HSD) test in a 2-way analysis of variance (ANOVA). [d]Entries for metabolites with highly characteristic NMR spectra that were not detected are indicated as less than their estimated upper detection limit (mM). [e]Metabolite concentration is significantly increased under NR versus fed conditions, as determined by Tukey's HSD test. [f]Note very high variance in metabolite concentration under NR. [g]Note very high variance in metabolite concentration.

correction was applied using common control points. Analyte peaks were aligned using *Icoshift*[21] and signals for background and internal controls were excluded. The spectra were normalized using PQN,[1] mean-centered and variance-stabilized using Pareto scaling.[22] PCA analyses were carried out using *PLS Toolbox* (Eigenvector Research Inc.).

The metabolite concentrations were measured using the *Chenomx NMR Suite* (Chenomx Inc.) according to the software's standard procedures. Briefly, the data sets were zero-filled, Fourier-transformed, phased and baseline-corrected in *Chenomx NMR Processor*. The chemical shape indicator was fitted to the DSS trimethylsilyl resonance and the spectra were transferred to *Chenomx NMR Profiler*. The library spectra were fitted in semiautomated fashion to the experimental data, yielding a read-out of the metabolite concentration relative to that of the known DSS standard.

The measurement uncertainties given in Table 1 were calculated using a two-way analysis of variance (ANOVA) test and significance was determined using the Tukey "honestly significant difference" (HSD) test.

With the design of the VDTS procedure in which identical volumes of standard ^{13}C-formate and DSS solutions are used for both real and control measurements, it can be shown (see Supporting Information) that the degree of dilution of the hemolymph into the ^{13}C-formate droplet can be written as

$$\frac{V_h}{V_f} = \left(\frac{I_c'^f}{I'^f} - 1\right) \quad (1)$$

where V_h is the volume of hemolymph sample released from the larvae, V_f is the (starting) volume of ^{13}C-formate solution in which the larvae are opened, and $I_c'^f/I'^f$ corresponds to the ratio of the ^{13}C-formate ^{1}H NMR peak intensities in the control (c) and real experiments and the primes indicate that each of the two spectra has been normalized to the DSS methyl group resonance. Knowledge of the dilution factor allows for extraction of the absolute concentration of metabolite X in the starting hemolymph according to (see Supporting Information):

$$[X]_h = \frac{I^X}{I^f}\frac{V_f}{V_h}[f]_0 \quad (2)$$

where $[f]_0$ is the concentration of ^{13}C-formate used in the droplet and I^X/I^f is the ratio of ^{1}H NMR resonance intensities measured for metabolite X and ^{13}C-formate, normalized to the number of protons contributing to the measured peak heights/areas (e.g., for each of the two ^{13}C-satellites of the ^{13}C-formate resonance this is 0.5 protons, for a nondegenerate methyl group resonance the corresponding value is three protons).

RESULTS

Obtaining NMR Spectra of Larval Hemolymph. Hemolymph was obtained by rupturing the cuticle of larvae in a droplet of saline of known volume. Clarified hemolymph was either diluted into D_2O containing reference standard DSS at fixed concentration (unextracted) or extracted for the polar components into an aqueous phase by a method that separates soluble metabolites from proteins and lipids,[19] followed by dilution into D_2O containing DSS (extracted). Consistent with other contexts, this extraction procedure was observed to recover >95% of the metabolites detectable in the unextracted data and quenches further chemical change. Despite the small amount of starting material, NMR spectroscopy of diluted hemolymph extracts from single larvae yielded data with reasonable S/N using signal averaging over a period of a few hours. High quality spectra could be obtained more quickly for batches of larvae, extracted into a single droplet sample. Figure 1 shows the 700 MHz ^{1}H NMR spectra of the extracted hemolymph polar metabolome for batches of fed (n = 10) and NR (n = 15) late L3 larvae using an overall data acquisition time of ~40 min. When normalized on a per-animal basis, the total metabolite signal intensity for the fed animals is significantly greater than that for the NR animals, presumably reflecting the lower body mass[5] in the latter cohort. By reference to databases of known metabolite NMR spectra, we identified the components present in each spectrum accounting for the vast majority (>90%) of the observed signals. Of note, a major component in the larval spectra corresponds to O-phosphotyrosine, easily distinguishable by the pair of doublet resonances in the aromatic region of the spectrum (δ = 7.17, 7.22 ppm). This metabolite is absent from all metabolite databases examined to date but, in *Drosophila*, it is known to be involved in sclerotization of the larval cuticle.[23−25] Consistent with this assignment, we did not find O-phosphotyrosine in the hemolymph metabolome of adult flies (data not shown). Another major component of the spectra corresponds to the disaccharide trehalose, α-D-Glu-(1→1)-α-D-Glu. Interestingly the contribution to the spectrum from glucose itself is extremely small, more than 2 orders of magnitude lower than for trehalose. This observation indicates that trehalose is the primary circulating form of sugar in larval hemolymph.

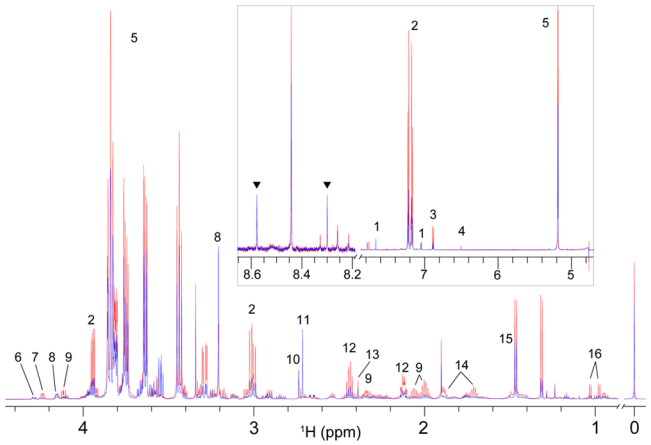

Figure 1. 700 MHz ^{1}H NMR spectra of the extracted hemolymph polar metabolome for batches of fed (red; n = 10) and NR (blue; n = 15) late L3 larvae *Drosophila* larvae. The trimethylsilyl resonance of DSS is set to 0 ppm. The downfield region of the spectra is displayed as an inset. The intensity scale is normalized to the number of larvae in each sample. The region between 8.20 and 8.65 ppm has been expanded vertically to visualize the H-1 doublet resonance of ^{13}C-formate indicated by black arrowheads. Resonances corresponding to select resonances are highlighted: histidine (1); O-phosphotyrosine (2); tyrosine (3); fumarate (4); trehalose (5); malate (6); threonine (7); phosphocholine (8); proline (9); sarcosine (10); dimethylamine (11); glutamine (12); succinate (13); lysine (14); alanine (15); and valine (16).

Absolute Metabolite Concentration by VDTS. Our aim was to characterize the impact of NR on the larval hemolymph metabolome in a quantitative manner. Obtaining the absolute metabolite concentrations requires normalization of the fed vs NR data on the basis of the extracted hemolymph volume. We obtained the extraction volume by indirect means, as described in schematic form in Figure 2. We denote this procedure as "volume determination using two standards" or VDTS. The larvae were ruptured in a precisely dispensed volume of saline containing a known concentration of ^{13}C-labeled sodium formate. A precisely measured portion of the clarified solution was diluted into water containing 80 μM DSS and submitted to chloroform−methanol extraction. The polar fraction was evaporated to dryness and resuspended in D_2O. The sample for analysis therefore contains both DSS and ^{13}C-formate as features that aid data set normalization (see Experimental Section). The DSS concentration is essentially constant for each sample, and the presence of the DSS methyl group resonance allows for both chemical shift referencing and normalization for any measurement variance arising from, for example, pipetting precision or variation in NMR probe sensitivity due to salt effects. The presence of the well-resolved ^{13}C-formate H-1 signal (doublet centered at δ = 8.44 ppm, $^1J_{CH}$ = 194.6 Hz) allows for the accurate and precise determination of the volume of hemolymph released into the droplet by comparison of the signal intensity to that obtained for a control droplet into which the same number of larvae have been introduced but not ruptured. Knowledge of the hemolymph starting volume allows for the derivation of the absolute

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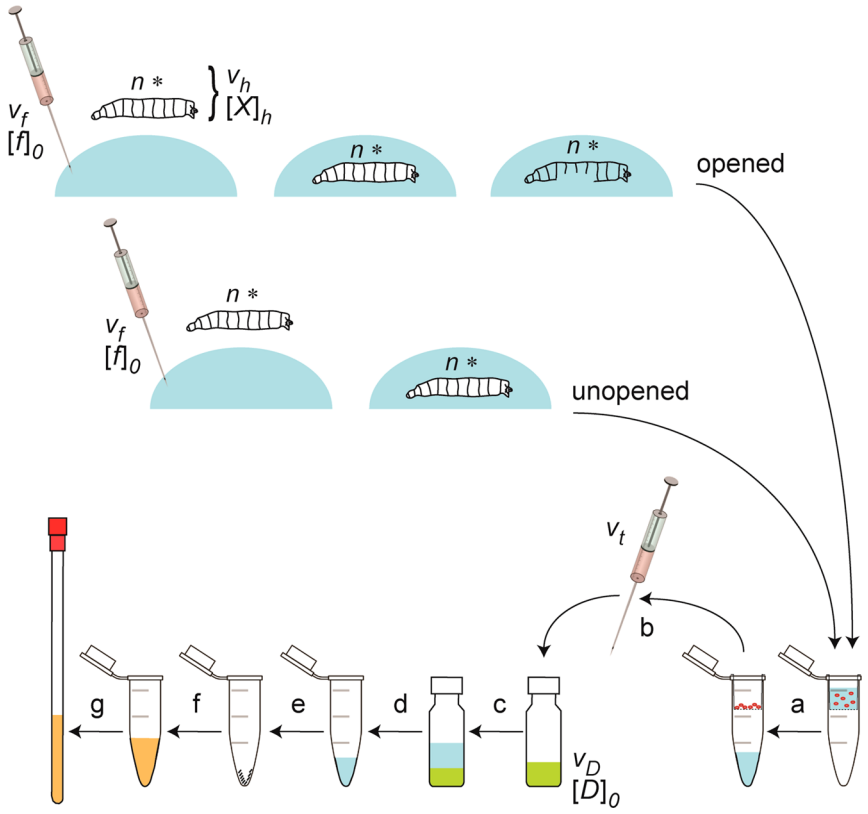

Figure 2. Outline schematic of the *Drosophila* hemolymph sampling strategy. A number (n) of larvae are either opened or unopened (control) in an accurately measured volume v_f of buffer containing ^{13}C-formate at a known concentration $[f]_0$. After centrifugal filtering to remove hemocytes (a), an accurately measured volume (v_t) of the diluted hemolymph is transferred to a known volume (v_D) of chloroform/methanol/water (green) containing a fixed concentration of DSS (b) in a glass vial. After addition of further extraction reactions (see Experimental Section), vortexing and centrifugation, the aqueous layer is aspirated to a microfuge tube (d). The solution is evaporated to dryness (e) and the residue suspended in D_2O (f) prior to transfer to an NMR tube (g). $[X]_h$: Concentration of metabolite X in neat hemolymph. v_h: Volume of hemolymph recovered.

concentration of the metabolites (*in the hemolymph*) from their NMR signal intensities normalized to the DSS standard (see Supporting Information).

Using the VDTS method, we found that hemolymph volumes from L3 larvae ranged over 692 ± 75 nL for fed females and 406 ± 34 nL for fed males, 234 ± 57 nL for NR males and 227 ± 64 nL for NR females. Using a database of metabolite NMR spectra (Chenomx Inc., supplemented with the spectrum for O-phosphotyrosine), which were collected with identical acquisition parameters,[6] we determined the absolute concentrations of 29 molecular components in the fed and NR data sets from male and female larvae. This procedure required highly supervised semiautomated fitting of the library with the experimental data. Table 1 lists the experimentally determined metabolite concentrations, calculated from triplicate samples in each of two completely independent fed vs. NR experiments. The reliably quantified metabolite concentrations range from ~70 mM down to ~0.05 mM. In addition, we could establish that a small number of other metabolites, that possess characteristic spectral features and are commonly detected in other systems, were definitively not detected in the hemolymph spectra. These include carnitine, cysteine, aspartic acid, glutamic acid, ornithine, and serine (see Table 1 for estimated limits of detection for these compounds). Within Table 1 bold figures indicate statistically significant differences *between males and females* for either the fed or NR conditions. The data in Table 1 demonstrate that, in fed larvae, the concentration of the majority of the quantifiable metabolites is significantly higher in male larvae than in females; a notable exception is glutamine. Under NR, the metabolite levels fall in both genders and the sex-dependent differences are greatly reduced (see Supporting Information Figure 1). However, the concentrations of two metabolites (betaine and phosphocholine) are elevated in both male and female NR larvae relative to the fed condition. For certain metabolites (e.g., malate) the levels are not detectably dependent upon gender or feeding status.

Impact of VDTS on Chemometric Analysis. The laborious nature of the fitting procedure used to obtain the absolute biological concentrations of all metabolites listed in Table 1 makes it unattractive to extend this approach to larger data sets (e.g., increased numbers of larvae; more dietary conditions; more developmental stages). Instead, it would be preferable to apply exploratory data analysis methods such as PCA to obtain similar insights into the differences between sample classes at a lesser time cost. However, the commonly applied preprocessing normalization procedures required for this approach such as PQN[1] implicitly assume that experimental manipulations (here fed vs NR, male vs female larvae) lead to small effects relative to the overall signal mass.

Figures 3 and 4 comprise the output of PQN/PCA analysis of the fed versus NR larval NMR data. The plots presented in Figures 3A and 4A were obtained by applying the analysis to the raw spectra without volume correction; the plots in Figure 3B and 4B are for the corresponding analysis when the individual spectra have been prescaled according to the dilution factors derived by measurement of DSS and ^{13}C-formate signal intensities using VDTS. In both cases, the PCA output (scores

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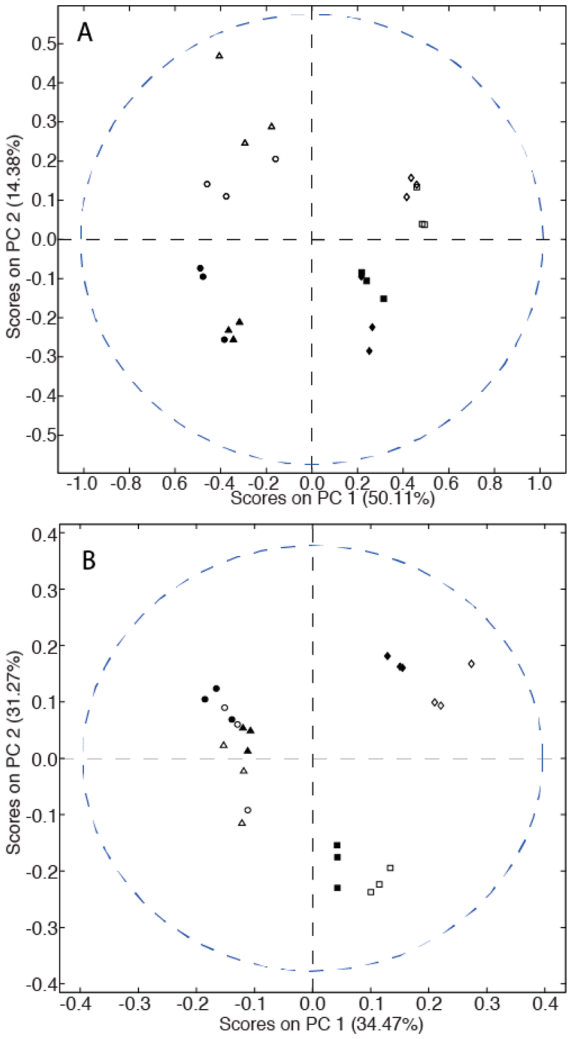

Figure 3. Scores plots from the PCA analysis applied to (A) NMR spectra that have been normalized using PQN and (B) NMR spectra that have been normalized on the basis of the V_h/V_f dilution factor by VDTS prior to intragroup PQN. Symbol key: diamond, fed males; square, fed females; triangle, NR males; circle, NR females. Open symbols represent data from the first of two independent experiments, filled symbols correspond to the second experiment.

plot; Figure 3) provides unambiguous discrimination of the fed and NR conditions along principal component (PC) 1. In the absence of volume correction, however, the loadings plot for PC1 possesses a negative, nonzero baseline offset across the entire spectral width (Figure 4A). In addition, the loading for PC2 shows signals of opposite sign for resonances from the same metabolite. This is most clearly demonstrated for the well resolved signals derived from the anomeric proton of trehalose at δ = 5.19 ppm. Figure 5 shows that one of the natural abundance ^{13}C-satellite peaks is negative, while the main ^{12}C-peak and other ^{13}C-satellite signal are both positive. The first of these outcomes indicates an intrinsic breakdown of the preprocessing procedure: nonzero baselines in loadings from mean-centered data indicate that the normalization step has failed. Therefore, without volume correction and under the assumption that the hemolymph volume sampled for each measurement is consistent, the loadings would be misinterpreted as the overall signal strength being much higher in fed versus NR larvae, leading to an overestimation of the differences between these conditions. The second outcome is not logically self-consistent as the loadings for different NMR signals for the same metabolite are not always "in-phase" nor with intensities characteristic of the relative number of protons in contributing to each multiplet. In addition, in the absence of volume correction, PCA erroneously separates the two independent experiments (via PC2) and fails to detect (via any of the first six PCs) the male vs. female separation that was clearly demonstrated with absolute metabolite concentrations (Table 1). In contrast, when VDTS dilution correction is applied, the PC1 loadings have essentially zero baseline offset, and all resonances for each metabolite exhibit consistent positive or negative direction (Figures 4B, 5). Following VDTS, PC2 also correctly provides discrimination between the males and females in the fed condition, while also indicating overlap of males and females under NR. These results show that accurate volume correction is required to obtain a logically consistent and biologically meaningful output for the multivariate analysis of the hemolymph metabolomics data set.

DISCUSSION

Utility of VDTS in the Metabolomics of Small Variable Sample Volumes. This study provides an optimized method for the sampling, quantitation and ^{1}H NMR analysis of metabolites in small, indeterminate and variable sample volumes. As a difficult test case, we sampled hemolymph from *Drosophila* larvae raised on well-fed vs. malnourished conditions. In this type of dietary manipulation, and probably in many other contexts, it is not valid to make the assumption that all samples yield comparable overall signal strength. Under these conditions, analyte volume differences must be taken into account after data acquisition in order to maximize the biological relevance of multivariate chemometric analysis. The VDTS protocol introduced in this study provides an accurate method for such volume corrections. In VDTS, one standard is used to normalize the spectra for any variance arising from measurement factors such as pipetting precision or variation in intrinsic spectrometer sensitivity from sample-to-sample. A second standard is then used to control for the degree of sample dilution into the analysis droplet. The characteristics of an ideal internal standard include that it should be chemically inert under the conditions of the measurement, possess signal(s) dispersed from those of other sample components, and not present in the biological samples. DSS is an industry-standard NMR reference compound for aqueous solutions that fulfills these requirements. The H-1 signal of formate satisfies only the first two of these criteria. Our experience has been that formate ions can be present, or adventitiously introduced, at detectable levels in analyte samples. Therefore we adopted ^{13}C-labeled-formate as the second standard: the $^1J_{CH}$ coupling splits the signal into a well-resolved doublet that does not overlap with any other metabolite that we have detected to date.

The VDTS protocol, combined with comparison of the NMR spectra with a database of library NMR spectra, permitted the determination of absolute concentrations of a large number of hemolymph polar metabolites under several different experimental conditions (see Table 1). Moreover, knowledge of the dilution factor (V_h/V_f) obtained for each sample using VDTS leads to a more biologically relevant PCA output than when the standard PQN normalization approach is applied to the uncorrected raw data. The aberrant nature of the loadings in the absence of correction for dilution factors (see Figure 4) reflects the application of mean-centering to data sets that contain high intensity signals with a high degree of variance, as is

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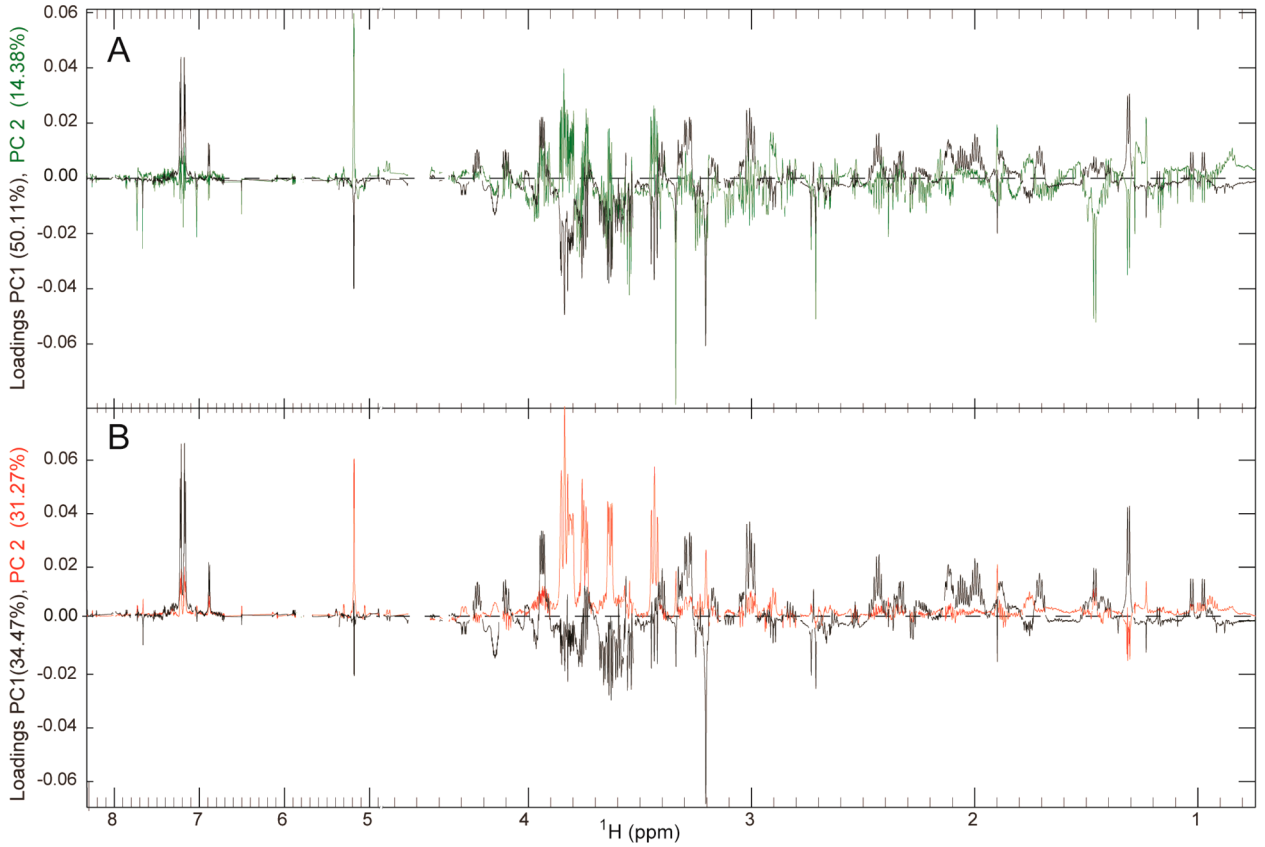

Figure 4. Loadings plots from the PCA analysis applied to (A) NMR spectra that have been normalized using PQN and (B) NMR spectra that have been normalized on the basis of the V_h/V_f dilution factor by VDTS prior to intragroup PQN. In each case PC1 is shown in black and PC2 in color (see Figure 5). Note chemical shift scale change at 5 ppm.

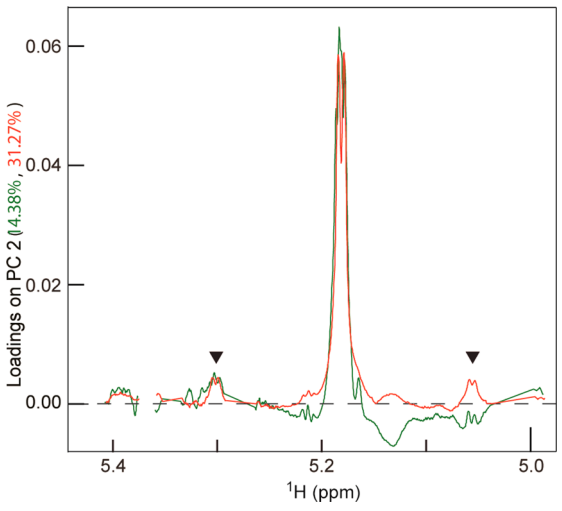

Figure 5. Expanded region of the PC2 loadings plot for NMR data that have been analyzed without (green) and with (red) VDTS (cf., Figure 4). Arrowheads highlight the natural abundance ^{13}C-satellites of the trehalose anomeric proton resonance at 5.19 ppm. The data analyzed with VDTS yields superior baseline characteristics and sensible in-phase and proportionate intensity.

often likely with small samples of indeterminate and variable volume. Importantly, the experimental logic of the VDTS approach has the potential to be employed in *any* context where the recovered analyte sample volume is so small as to be indeterminate with a high degree of precision. As we have demonstrated, application of the VDTS protocol allows for a more sensible output of the PCA process for different conditions, leading to a logically self-consistent loadings plot during multivariate analysis of the data, and the reliable identification of important features in the data.

Comparison of VDTS-NMR with Other Polar Metabolomic Approaches Used in *Drosophila*. Given the widespread use of *Drosophila* as a model organism, and the relative ease of dietary and tissue-specific genetic manipulation, there is surprisingly little in the literature by way of comprehensive metabolomics investigations in this species. This presumably reflects the practical complexities associated with limited sample volumes. Nevertheless, a small number of studies report targeted measurements of amino acids and other compounds in hemolymph using hybrid liquid chromatography (LC)-MS and capillary electrophoresis-laser induced fluorescence (CE-LIF) procedures.[26−32] In particular Shippy and co-workers have demonstrated a method to detect and estimate the concentration of a subset of amino acids in both larval and adult hemolymph of single animals, using chemical derivatization and CE-LIF.[26−28] Further discussion of issues relating to metabolomics measurements in small volume samples is provided in the Supporting Information.

In many insect species, the nonreducing carbohydrate trehalose is the major blood sugar.[33] Interestingly, with VDTS-NMR we detect only a very low level of circulating glucose (~0.2 mM glucose vs ~40 mM trehalose), which is in contrast to other reports in the literature.[34] While variations in reported glucose concentrations may partially reflect changes in diet or larval stages, postharvesting degradation of trehalose to glucose in

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nonextracted hemolymph appears to be a major problem (see Supporting Information Figure 2). This is likely due to the persistence of active trehalase enzyme in the hemolymph.[35] However, when hemolymph is rapidly extracted with methanol-chloroform, we find that the trehalose signal is stable over time. The artifactual appearance of glucose in the hemolymph has important implications for the interpretation of metabolic studies in *Drosophila*, and we suggest that care should be taken to suppress adventitious trehalose breakdown in future studies. In a similar fashion, we also detected rapid diminution of the tyrosine signals in unextracted hemolymph samples, which could be attributable to an artifactual melanization reaction (see Supporting Information Figure 2).

We could not detect glutamate ions in our NMR spectra of hemolymph, which contrasts with other reports suggesting a range of millimolar concentrations for this amino acid.[26,27,36,37] Some of these previous studies[27,28] used an extraction technique involving dried, pinned larvae that were dissected on a microscope stage and the hemolymph withdrawn via a capillary of large diameter (relative to the animal). We reasoned that opening of the larva by cuticle rupture in a buffer droplet is a more rapid method of hemolymph extraction that minimizes the stress to the animals prior to extraction. We note that circulating glutamate ions would probably need to be kept low in the hemolymph, given that this molecule is a neurotransmitter at the *Drosophila* neuromuscular junction.[38] Interestingly we were easily able to detect glutamate in whole larval extracts, that is, when larvae were harvested by ball bearing homogenization rather than careful tearing with forceps (data not shown). These observations suggest that glutamate is compartmentalized in *Drosophila* tissues other than hemolymph. Using the CE-LIF method, the larval hemolymph concentrations of other amino acids were also calculated by Piyankarage et al.[27] We note that the degree to which the reported concentrations might correspond to our measurements is difficult to assess since the developmental stage in that study was not more precisely defined than "third instar", and the sex was not reported. Nevertheless, the overall profile of amino acid concentrations is broadly consistent with our observations. Advantages of the VDTS-NMR method are that it can capture all polar metabolites, not just amino acids, and it is not compromised by hemolymph evaporation nor by any potential errors derived from sample volume calculations using the assumed internal diameter of the Tygon-tubing/capillary collection device.

Further investigations will be required to reveal the biological mechanisms underlying the surprising and marked sex-specific differences in larval hemolymph that were observed in this study. However, we note that the average larval masses of fed males compared to fed females and fed vs NR animals[5,39] appear to correlate with the VDTS-derived hemolymph volumes. It is also interesting that female larvae grow at a higher rate than their male counterparts and attain a larger pupal mass. Hence it is possible that females deplete their hemolymph nutrients more substantially than males during the larval growth period. Consistent with this speculation, when larval growth is equalized close to zero for both genders during NR, most sex-specific differences in metabolite concentrations are lost.

CONCLUSION

We have applied ^{1}H NMR spectroscopy to the analysis of polar metabolites in *Drosophila* larval hemolymph under two different dietary regimes. In order to obtain a properly meaningful multivariate analysis of the sex- and diet-dependent measurements, we developed a practical procedure (VDTS) that allows for the precise and accurate determination of the analyte starting volumes. Based upon these volume measurements, we could establish absolute metabolite concentrations under each condition. We propose that VDTS therefore provides a useful tool for further study of small volume mixtures by ^{1}H NMR, and is particularly beneficial when the presumption of conservation of spectral mass is questionable. We predict that, with minor modifications, the VDTS procedure could be usefully adapted to other measurement methods such as LC-MS.

ASSOCIATED CONTENT

Supporting Information

Additional figures, derivation of equations, and commentary as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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T.J.R., A.P.B., A.P.G., and P.C.D. conceived and designed the experiments. T.J.R. and A.P.B. performed the experiments. All authors wrote the manuscript and have approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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