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Between-person comparison of metabolite fitting for NMR-based quantitative metabolomics

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TITLE RUNNING HEAD. Between-person comparison of metabolite deconvolution.

ABSTRACT. Nuclear magnetic resonance (NMR) spectroscopy is widely used as an analytical platform for metabolomics. Many studies make use of 1D spectra, which have the advantages of relative simplicity and rapid acquisition times. The spectral data can then be analysed either with a chemometric workflow, or by an initial deconvolution, or fitting, step to generate a list of identified metabolites and associated sample concentrations. Various software tools exist to simplify the fitting process but at least for 1D spectra, this still requires a degree of skilled operator input. It is of critical importance that we know how much person-to-person variability affects the results, in order to be able to judge between different studies. Here we tested a commercially-available software package (Chenomx' NMR Suite) for fitting metabolites to a set of NMR spectra of yeast extracts, and compared the output of five different people for both metabolite identification and quantitation. An initial comparison showed good agreement for a restricted set of common metabolites with characteristic well-resolved resonances, but wide divergence in the overall identities and number of compounds fitted; re-fitting according to an agreed set of metabolites and spectral processing approach increased the total number of metabolites fitted, but did not dramatically increase the quality of the metabolites that could be fitted without prior knowledge about peak identity. Hence, robust peak assignments are required in advance of manual deconvolution, when the widest range of metabolites is desired. However, very low concentration metabolites still had high coefficients of variation even with shared information on peak assignment. Overall, the effect of person was less than experimental group (in this case, sampling method) for almost all metabolites.

KEYWORDS: NMR spectroscopy, metabolomics, metabonomics, targeted profiling, quantitation, deconvolution

ABBREVIATIONS: 3M2OV, 3-methyl-2-oxovalerate; AMP, adenosine monophosphate; NAM, β-

nicotinamide mononucleotide; Met. sulfox., methionine sulfoxide; NAD+, nicotinamide adenine dinucleotide; NADP+, nicotinamide adenine dinucleotide phosphate; UDP, uridine diphosphate; GlcNAc, *N*-acetylglucosamine; sn-G3PC; sn-glycero-3-phosphocholine.

Introduction.

Nuclear magnetic resonance (NMR) spectroscopy is very widely used for metabolic profiling (metabolomics), [1] [2] and has been the subject of major investments such as the Human Metabolome Database [3]. This continued popularity is due to a number of inherent properties of NMR – in particular, proton NMR is a near-universal detector, and reports on metabolites from all chemical classes simultaneously, with high precision [4] [5]. Furthermore, because there is no physical separation of metabolites, and because, disregarding relaxation effects, the intensity of a resonance is proportional to the actual numbers of nuclei giving rise to it, NMR is particularly powerful for making between-metabolite comparisons, and can detect even subtle relative concentration changes [6].

There are two distinct classes of methods used for analysing NMR metabolomic data. Chemometric approaches use pattern-recognition methods to analyse whole spectra, and individual metabolites need only be identified *a posteriori* following identification of discriminatory peaks [7]. Conversely, deconvolution or metabolite fitting methods aim to match resonances to standards based on known molecules. This has been referred to as "quantitative metabolomics" [8] or "targeted profiling" [9]. Chemometric methods have several advantages – in particular, that spectra can be analysed without prior knowledge of what metabolites are present, even for wholly novel compounds. However there are also many advantages to the quantitative metabolomics approach. Firstly, the data analysis problem is reduced from hundreds or thousands of bins/data points (with unhelpful statistical properties such as high correlation between variables) to, typically, tens of variables that represent actual discrete entities. Secondly, knowledge of chemical identities permits analyses based on prior knowledge – for instance, chemical similarity, or pathway relatedness [10] [11] [12]. Thirdly, quantitative data are much more valuable if metabolomic data are to be used as part of an input for a systems biology study. Finally, if studies are to be compared between different labs – an essential underpinning of science – then quantitative data on named metabolites are ideal.

There are a number of different software options for fitting metabolites to NMR data, reviewed by Wishart[8]. In our own lab, we have made use of a commercial package, NMR Suite (Chenomx, Edmonton, Canada). This software is widely used: as of July 2011 and since 2007 there are over 220 papers with "Chenomx" as a search term based on a full-text search of papers from seven major scientific publishers (Wiley, Elsevier, ACS, Springer, RSC, Nature.com, and BiomedCentral). Clearly this is not a comprehensive search and there will be many additional studies from other publishers. The NMR Suite software is not completely automated (although its use in fully-automated studies has recently been explored [13]): rather, it provides computer-assisted manual fitting. This is both an advantage (because it allows individual judging of peak shifts and shapes by the analyst) and potential disadvantage (because there is then an element of subjectivity in the assessment). When a single person is responsible for the fitting, the reproducibility is very good: independently-fitted technical repeats are more similar to each other even than to other samples within the same control group [10]. However, in order to be able to compare between studies, one needs to know the between-person reproducibility. Surprisingly, there is as yet no published evaluation of this.

We compared the NMR Suite software between five different analysts, and report here how the reproducibility varied across different metabolites. In order to obtain information on the widest possible range of metabolites, it was necessary to share information on peak assignment; however, a core group of easily-assigned and high-concentration metabolites compared well between different analysts even with no prior sharing of information.

Methods.

We took spectra from an earlier study on the bioprocessing yeast *Pichia pastoris* [14]. The data set consisted of six classes, representing cells sampled by one of six different methods: unquenched (centrifuged) cell extracts (OX); cell extracts made with four different methods based on rapid quenching with cold solvents (QXA, QXB, QXC and QXD); and a total quenched extract (TX) of both

cells and broth combined. Each class contained three replicates, giving a total of 18 spectra. All participants then fit these data independently in two rounds. In round one, to mimic completely independent analysis, all participants processed the spectra and then fitted metabolites using NMR Suite 6 (Chenomx, Edmonton, Canada) completely independently. Four of us chose to process and fit the spectra within NMR Suite, while one processed the spectra using iNMR 3 (Nucleomatica, Molfetta, Italy), with export as JCAMP files and subsequent import of the processed files into NMR Suite. Metabolite assignment was carried out individually, using any available resources (i.e. online data sources such as BioMagResBank [15] and HMDB [3, 16] could be used in addition to the NMR Suite internal database, if desired). The only guidance given on metabolite assignment in round one was that additional standard files for two high-concentration metabolites (trehalose and arabitol) were shared with all participants.

In round two, we shared both processed spectra and "template profiles" (i.e. previous examples of spectra fitted using NMR Suite), in order to represent the situation where outside information on metabolite assignment was available. The spectra were processed using iNMR 3 (with a zero-filling factor of two and exponential line broadening of 0.5 Hz), and exported as JCAMP files, which were then given to all participants. The processing was fully automated (using the iNMR software routines for metabolomic phase correction, baseline correction using a smoothing function [17] and referencing chemical shifts to the internal trimethylsilyl resonance), and so we did not consider it necessary to have this step repeated by individual participants. The spectra were then fitted a second time in NMR Suite after first importing one of two template profiles for each spectrum (one for all TX samples, and one for all remaining samples). This ensured that all participants fitted exactly the same set of 37 metabolites (Table 1) to the spectra. (Assignment of the metabolites, including 2D NMR and spiking of authentic standards, is described in Tredwell et al. [14].) In addition, all participants viewed the fitting of one spectrum together, in order to try and normalize this subjective process between participants.

Data analysis was carried out using Matlab (Mathworks, Massachusetts, USA) and Aabel 3 (Gigawiz, Tulsa, USA). The spectra can be downloaded (both as raw and processed files) from the online supplementary information.

Results and discussion.

In round one of the metabolite fitting, participants were asked to process the NMR spectra (such as phasing and baseline correction) independently. This was the first time the majority of the participants had seen samples of this particular composition and the only information given regarding metabolite assignment was that two high concentration metabolites, arabitol and trehalose, were present. From round one, a total of 77 metabolites were fitted in at least one sample (Fig. S1, supplementary information). However, a number of these assignments were clearly tentative, having been fitted to a small number of samples, and while the majority of metabolite assignments were fitted to all samples, not all people had made the same assignments. When compared across all five of us, just 16 metabolites were fitted in almost all cases (Fig. S1, online supplementary information), and so we initially analysed the data from these 16 compounds only (Table 1).

In round two, all participants re-fitted all spectra, but using automated routines for spectral processing and baseline correction so that the processing was identical for all people, to exclude any person-to-person variability in the processing step. In particular, there are likely to be differences in baseline correction between people. Broad underlying signals from macromolecules such as proteins and lipids are a common feature of metabolomic samples, which makes identifying the baseline a difficult process. This can also become quite a subjective process, if one chooses to manually fit spline curves to take into account these underlying signals.

In addition to the 16 metabolites assigned from round one, a further 21 metabolites, for which assignments had been confirmed by 2D NMR and spike-in experiments, were included in round two

(Table 1). The full metabolite list was saved as a Chenomx "template" profile, and participants were instructed to load these profiles before fitting. This ensured that all metabolites were included in round two by all participants.

We initially used principal components analysis (PCA) to give a quick visual comparison of the overall variability of different participants (Fig. 1). For the round one data, the samples clustered by extraction method along PC 1, with the total quenched (TX) samples clustering near the quenched samples (QX), a separate cluster for the unquenched samples (OX), and an outlying sample (QXB1), which had losses during sample preparation and as a result contained low concentrations for all metabolites. PC2 shows a clear separation of person 2 (Fig. 1a), with the loadings indicating differences in threonine. Inspection of the fitted spectra revealed that person 2 had fitted lactate as well as threonine to a doublet resonance at 1.32 ppm. This instance highlights the potential of user bias when processing new sample types and reinforces the need for 2D NMR spectra for assignment confirmations. The most characteristic resonance for both lactate and threonine is a doublet at 1.32 ppm, and while other smaller signals at 3.58 and 4.27 ppm for threonine, and 4.10 ppm for lactate can assist in correct assignment, these regions in the current sample set are complex with many underlying signals, and 2D NMR experiments are the most reliable way to distinguish between the two compounds. In the current study lactate was actually present but only in low concentrations, too small to affect the fitting of threonine.

When threonine and the outlying sample QXB1 were excluded from the PCA analysis (Fig. 1b) there were no longer any clear differences between people (Fig. 1b), and data points were largely clustered by sample type in both PC1 and PC2, with the TX and QX samples overlapping slightly.

The data for round two clustered largely by sampling method and not by person, and again sample QXB1 was a clear outlier (Fig. 1c). With this sample excluded, there was very clear clustering according to sample method rather than by person (Fig. 1d). It should be borne in mind that the current study is of a very controlled set of samples: yeast cells that had all been grown under the same conditions, and differed only in the methods used to sample cells for extraction. This therefore

represents a very conservative comparison between people and experimental treatments, and it is likely that the results would be even clearer if we had used highly variable samples such as urine or cell growth media.

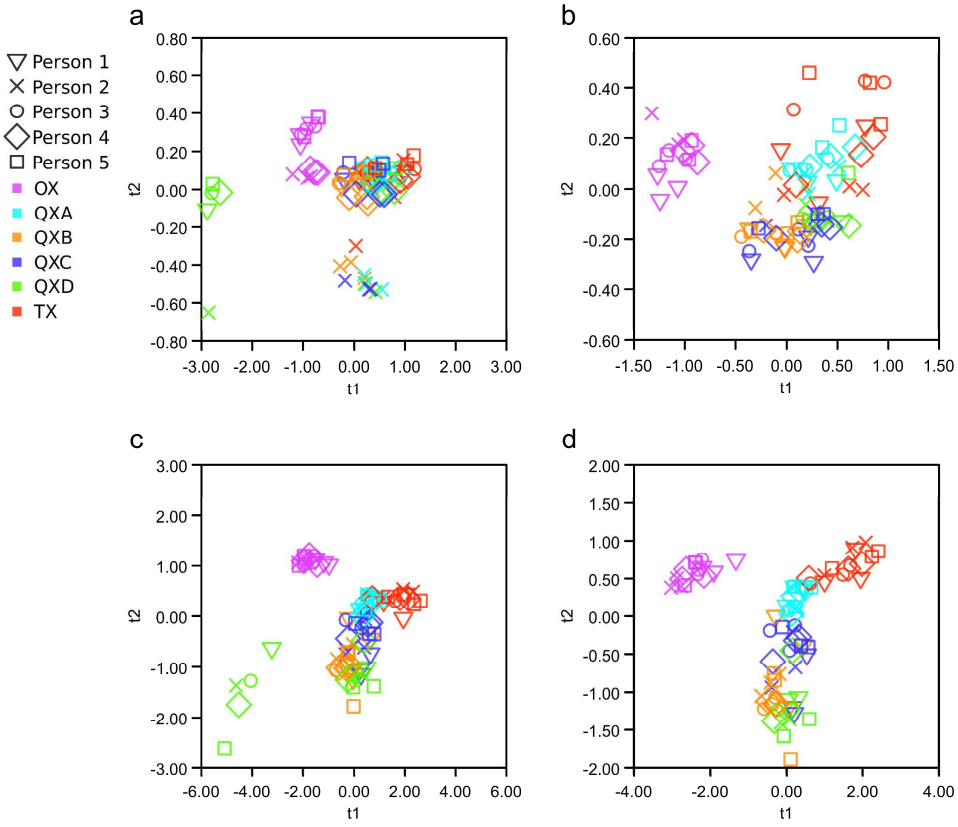

Figure 1 Principal component analysis scores plots of a) 16 metabolites fitted independently from round one; b) As for (a), but with one metabolite (threonine) and one outlying sample (QXB1) excluded; c) 37 metabolites fitted based on exemplar spectra in round two; d) As for (c), but with one outlying sample (QXB1) excluded. All datasets were log transformed and mean centered prior to analysis.

The multivariate analysis of both rounds one and two indicates that the biological variation between samples was greater than the person-to-person variation of metabolite fitting when considering the data simultaneously, since there was clustering of sample methods rather than of different people. We also assessed this using two-way analysis of variance (ANOVA) for each metabolite in turn, with experimental treatment ("method") and individual variability in fitting ("person") as factors. The results for round two are shown in Table 1. Seventeen out of 37 metabolites had a non-significant "person" effect, i.e. all five participants fitted the metabolites effectively the same. This means that 20 out of 37

metabolites had significant differences in fitting for at least one of the participants. This may sound like a poor outcome; however we can also use ANOVA to compare the relative contributions of "method" and "person". For round one, "method" had a greater contribution to variance than "person" for all metabolites except threonine, which had already been shown to be problematic by PCA (Table S1, supplementary information). For round two, three metabolites – malate, nicotinate and UDP-glucose – were more affected by person than sampling method, but this still means that for the large majority of 34 compounds, the relative effect of individual analyst variability was smaller than experimental treatment, even for highly controlled and very similar samples.

Table 1. Metabolites fitted in the current study, and comparison of the contribution of individual variability in fitting (factor "person") and experimental treatment (factor "method") on variance; ANOVA carried out on log-transformed data from round two, i.e. with shared information on metabolite assignment. One outlying sample (QXB1) was excluded from the analyses. [a]Metabolites fitted in both rounds one and two. PCID: PubChem ID.

		Method			Person			Interaction		
Metabolite	PCID	Mean Square	F	p	Mean Square	F	p	Mean Square	F	p
3M2OV	47	5.02	186	<0.001	0.077	2.86	0.067	0.026	0.980	0.497
AMP	6083	1.16	18.3	<0.001	0.11	1.79	0.146	0.091	1.44	0.145
Alanine	5950	0.027	4.48	0.002	0.0013	0.212	0.930	0.0013	0.0504	1.000
Arginine	6322	0.56	96.4	<0.001	0.0075	1.29	0.285	0.0011	0.186	1.000
Asparagine	6267	0.29	67.8	<0.001	0.023	5.27	0.001	0.0046	1.08	0.386
Aspartate	5960	0.93	139	<0.001	0.020	2.97	0.027	0.0022	0.325	0.996
NAM	14180	0.18	6.11	<0.001	0.070	2.37	0.081	0.062	2.10	0.018
Citrate	311	0.45	17.6	<0.001	0.13	5.15	0.001	0.039	1.51	0.115
Met. sulfox.	847	0.31	59.1	<0.001	0.0080	1.52	0.208	0.0014	0.0850	1.000
D-Arabitol	94154	0.41	53.5	<0.001	0.0077	1.01	0.409	0.0020	0.125	1.000
D-Trehalose	7427	0.40	71.6	<0.001	0.0018	0.151	0.962	0.0012	0.226	1.000
Formate	284	1.00	60.4	<0.001	0.15	9.33	<0.001	0.040	2.42	0.007
Fumarate	444972	3.86	47.5	<0.001	0	0	NaN	0.11	1.33	0.234
Glucose	5793	2.08	137	<0.001	0.040	2.65	0.043	0.0051	0.337	0.995
Glutamate	33032	0.22	41.6	<0.001	0.0027	0.503	0.734	0.0046	0.857	0.637
Glutamine	5961	0.29	31.9	<0.001	0.013	1.49	0.216	0.0023	0.252	0.999
Histidinol	776	1.32	310	<0.001	0.014	3.26	0.018	0.0018	0.418	0.983
Isoleucine	6306	0.25	35.8	<0.001	0.069	9.83	<0.001	0.023	3.26	<0.001
Leucine	6106	0.26	37.2	<0.001	0.028	4.04	0.006	0.0044	0.637	0.866
Lysine	5962	0.53	46.0	<0.001	0.088	7.58	<0.001	0.011	0.993	0.484
Malate	525	0.048	2.35	0.066	0.28	13.6	<0.001	0.043	2.08	0.019
NAD+	5893	0.28	28.2	<0.001	0.069	6.87	<0.001	0.0044	0.436	0.978
NADP+	5886	1.16	11.4	<0.001	0.92	9.06	<0.001	0.12	1.23	0.263
Niacinamide	936	0.28	5.03	0.002	0.23	4.03	0.013	0.057	1.02	0.459
Nicotinate	938	0.31	4.37	0.005	0.79	11.0	<0.001	0.15	2.10	0.021
Phenylalanine	6140	0.54	51.6	<0.001	0.043	4.07	0.006	0.025	2.40	0.005
Pyroglutamate	7405	1.20	86.8	<0.001	0.019	1.38	0.253	0.0094	0.676	0.832

Serine	5951	0.39	19.9	<0.001	0.22	11.5	<0.001	0.024	1.21	0.276
Succinate	1110	0.66	8.67	<0.001	0.10	1.34	0.267	0.077	1.01	0.464
Threonine	6288	0.24	38.2	<0.001	0.053	8.40	<0.001	0.0039	0.619	0.881
Tryptophan	6305	0.77	21.9	<0.001	0.23	6.44	<0.001	0.11	3.04	0.001
Tyrosine	6057	0.47	63.1	<0.001	0.0058	0.789	0.537	0.0028	0.379	0.990
UDP-GlcNAc	16667373	0.47	41.5	<0.001	0.026	2.28	0.072	0.0090	0.795	0.708
UDP-glucose	8629	0.024	0.56	0.691	1.39	33.1	<0.001	0.079	1.89	0.038
Valine	6287	0.17	30.0	<0.001	0.0093	1.63	0.180	0.0026	0.453	0.973
sn-G3PC	439285	0.49	75.6	<0.001	0.022	3.34	0.016	0.0015	0.232	1.000
trans-Aconitate	444212	0.39	2.41	0.080	0.073	0.446	0.643	0.15	0.923	0.553

To look more closely at differences between people when fitting metabolite concentrations, we first performed pairwise rank correlations (Spearman's ρ) of individual metabolite concentrations over all 18 samples, and secondly, pairwise correlations of individual samples over all metabolites (i.e. 16 for round one and 37 for round two). With five participants this results in ten pairwise comparisons (Fig. 2). For round one it is clear once again that person 2's values for threonine concentrations do not agree with the other participants, and this is also evident for phenylalanine (Fig. 2a). Apart from these two metabolites however, pairwise correlations for metabolites are high across all participants. While person 2 clustered separately for round one, we see that a different participant, person 1, clusters separately for round two (Fig. 2a). For round two the majority of metabolite correlations are good, especially for the same 16 metabolites fitted from round one. However, a number of metabolites do not correlate well between people. While these correlations are much worse for comparisons with person 1, they are still below average for all participants, with malate showing the worst correlations between people.

The correlations of individual samples between people (ρ) are much higher than for the comparison of metabolites (Fig. 2b). Once again we see that person 2 from round one and person 1 from round two clustered separately. Generally the TX samples, which are slightly more complex than the other samples due to the presence of extracellular metabolites and media components, and the QXB1 sample, which contained low concentrations for all metabolites, resulted in slightly lower correlations.

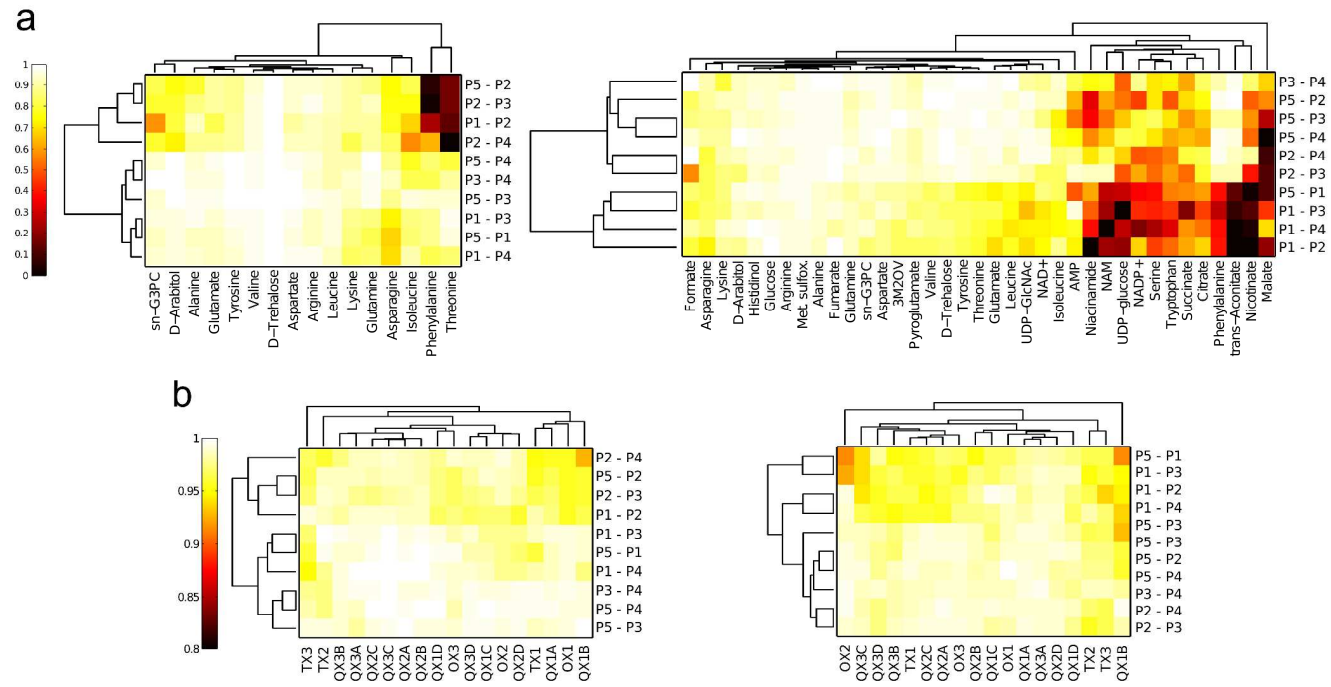

Figure 2. Correlations (Spearman's ρ) between people for both metabolites and samples ordered by hierarchical clustering in both dimensions. a) association between different people for individual metabolites, 16 metabolites fitted independently from round one (left hand side) or 37 metabolites fitted based on exemplar spectra from round two (right hand side); b) association between different people for individual samples. 16 metabolites fitted independently from round one (left hand side) or 37 metabolites fitted based on exemplar spectra from round two (right hand side).

Finally, to investigate the variance across all five participants, we calculated mean concentrations and percent coefficients of variation (%CV) between people (n=5) for each metabolite in individual samples. This gave 288 data points (18 samples × 16 metabolites) for round one and 666 data points (18 samples × 37 metabolites) for round two (Fig. 3). A comparison of the same 16 metabolites for rounds one and two shows that there was no real improvement in the data resulting from the prior sharing of information, with the majority of data points below 20% coefficient of variation for both rounds (Fig. 3a). When including all of the metabolites from round two, however, the data appear worse, as the distribution of %CV is higher (Fig. 3b). We wanted to know if this was because round two was largely adding lower-concentration metabolites, which could not be fitted as precisely, and so we plotted mean metabolite concentrations (n=18, i.e. average across all spectra) against mean %CV for both rounds one and two (Fig. 3d and 3e respectively). There is clearly a relationship between metabolite concentration and between-person variability in fitting, and it was indeed the case that round two added a number of

metabolites with very low concentrations and concomitantly high %CV. However, there were also seven additional metabolites in round two with mean coefficient of variation < 20%, and three additional high-concentration metabolites that nonetheless had relatively poor %CV (citrate, malate, and serine). In these cases, the greater variability can be attributed to the fact that their resonances appear in highly complex regions with overlapping signals from other metabolites. Malate was difficult metabolite to quantify in these spectra due to overlapping resonances from usually higher concentrations of aspartate, glutamate and pyroglutamate. Citrate was overlapped with malate, aspartate, pyroglutamate, and other unassigned resonances. Serine was partially overlapped with resonances from several other high-concentration compounds, especially arabitol, the highest-concentration metabolite in *P. pastoris*.

What implications do these results have for metabolomics studies in general? Some of the fitted metabolites have extremely poor reproducibility across different individuals (Figs. 2 and 3). This seems at first far worse than similar studies of 1D spectra of replicate samples, which have reported a median CV of around 3% [18] – but it must be remembered of course that these are the extreme values. The median %CV for round two (Fig. 3b) was 14% which, while still high, is much more acceptable, particularly when it is remembered this represents a between-person metric. It should also be borne in mind that a majority of studies will probably have data fitted by one person only. In order to compare to this common situation, we had one participant fit one spectrum five times independently. Unsurprisingly, the results were far more precise, with a median %CV of just 2.4%, and almost all metabolites (32 out of 37) below 10% (Fig. 3c). Our between-person comparisons will likely be more relevant to comparing between different studies. Such between-laboratory comparisons have been made for NMR metabolomic data [19] [20], and indicate that the between-person contribution to variation is not greater than the typical between-lab contribution (and the within-person contribution is less than both).

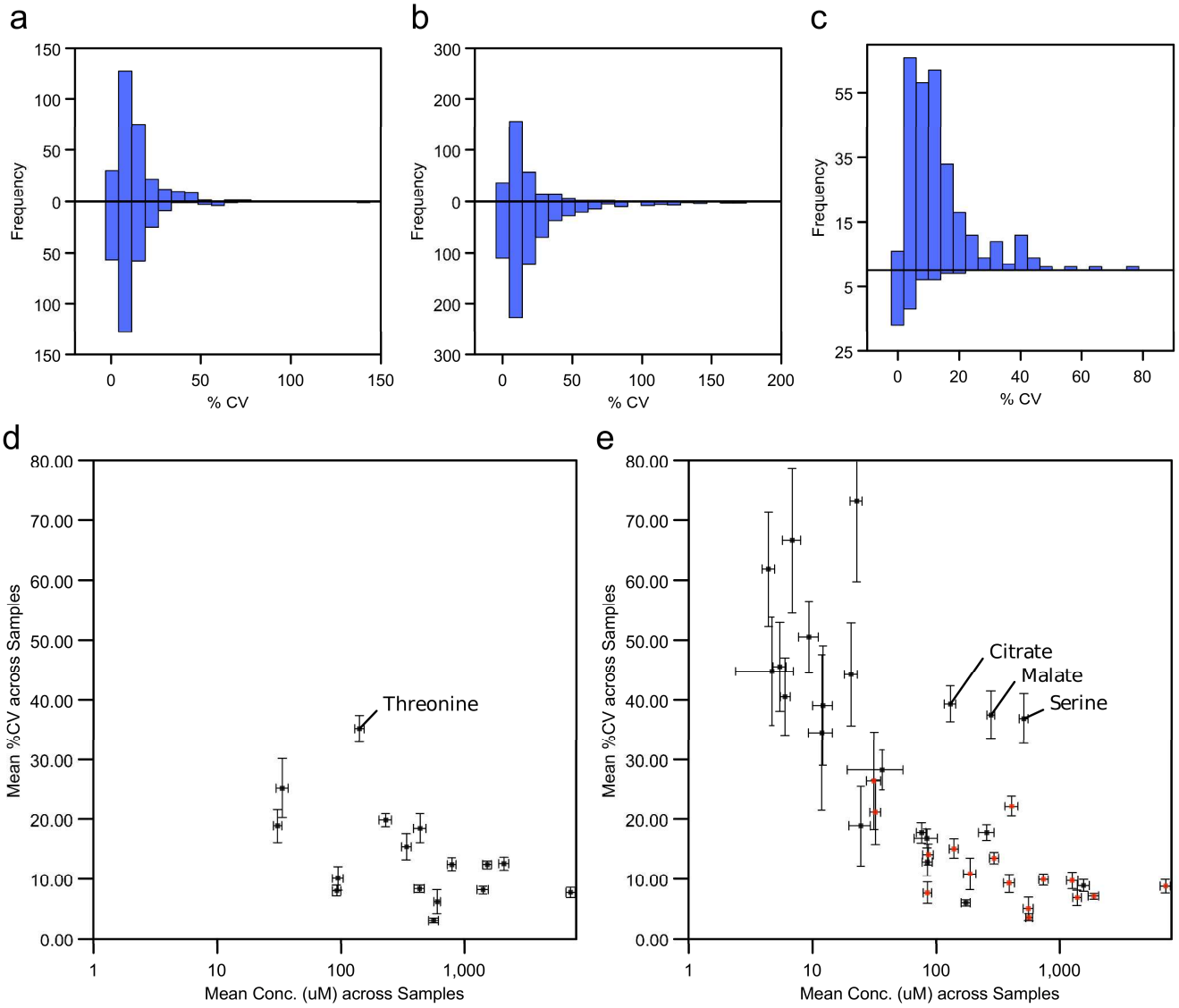

Figure 3. a) Distribution of coefficients of variation between people, calculated for each metabolite for each spectrum separately (n=5). Comparison of 16 metabolites fitted independently from round one (top) to the same 16 metabolites fitted based on exemplar spectra from round two (bottom); b) comparison of 16 metabolites fitted independently from round one (top) to 37 metabolites fitted based on exemplar spectra from round two (bottom); c) comparison of 16 metabolites fitted independently from round one (top) to the within-person variation of 37 metabolites fitted in one spectrum five times independently. d) Relationship between mean metabolite concentration and mean coefficient of variation for 16 metabolites fitted independently from round one across samples; e) as for (d) but for 37 metabolites fitted based on exemplar spectra from round two. Red data points refer to the same 16 metabolites as in (d).

To summarize, given the large number of studies that make use of manual, quantitative, targeted metabolite assignments, it is important to characterize the reproducibility between different people. Overall we found a good agreement between fitted metabolite concentrations amongst five analysts, certainly good enough to permit comparisons of studies between different people or even between different groups. The main sources of variation were incorrect assignments, overlapping signals, or low

metabolite concentrations, although generally this variation was still less than the biological variation, even for a set of very similar cell extracts. Sharing prior information on spectral assignment enabled reproducible fitting of a larger number of metabolites, but the differences in spectral processing between people were not a large source of error.

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Supporting Information Available.

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