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Automated annotation and quantification of metabolites in ^1H -NMR data of biological origin

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Abstract

In metabolomic ^1H -NMR datasets, there are often over a thousand peaks per spectrum, many of which change position drastically between samples. Automatic alignment, annotation and quantification of all the metabolites of interest in such datasets have not been feasible. In this work we propose a method which requires annotation of metabolites only in a single spectrum. The annotation database built from that single spectrum can then be used as a basis for fully automated annotation and quantification in any number of ^1H -NMR datasets with similar background matrix.

The method is based on the Generalized Fuzzy Hough Transform (GFHT) for alignment and on Principal Component Analysis (PCA) for peak selection and quantification. We show that we can establish quantities of 20 metabolites in several ^1H -NMR datasets and that the method is extendable to include any number of metabolites that can be identified in a single spectrum. The method speeds up the quantification of previously known metabolites and also returns a table containing the intensities and locations of all the peaks that were found and aligned but not assigned to a known metabolite. This enables both biopattern analysis of known metabolites and data mining for new potential biomarkers among the unknowns.

Keywords

^1H -NMR; alignment; multivariate; metabolomics; Hough transform; urine; quantification; spectral profiling

Introduction

Metabolomic analysis of biological samples using high-resolution ^1H -NMR spectroscopy is an active field of research[1]. ^1H -NMR as a technique has the advantage of providing reproducible quantification results[2, 3], while the main disadvantages are peak overlap and a rather high limit of detection. Much of the effort in the field has been focused towards alignment[4–7] or binning[8–11] of spectra followed by chemometric analysis utilizing

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methods such as Principal Component Analysis (PCA)[11–13] and Partial Least Squares Discriminant Analysis (PLS-DA)[14]. In recent years however, direct quantification of metabolites from spectral data has grown in popularity[15]. To make high throughput quantification possible, development of automated methods for this purpose is required.

Automated quantification of metabolites from 1-D ^1H -NMR data of complex biological samples is a challenging task. Peak misalignments and peak overlap in crowded regions of the spectra are problems that are not easily overcome[16]. Differences in the physical and chemical properties of e.g. urine samples cause peaks to shift as much as up to 0.1 ppm between analysis runs even in samples with added pH buffers and measured at controlled temperatures. At the same time, ^1H -NMR spectra often contain as many as a thousand peaks, creating a lot of overlap and background signal in crowded regions. On the other hand, each analyte gives rise to several signals in ^1H -NMR spectra, which creates a possibility to select the peaks that carry the highest quality information; i.e. has the least overlap and highest signal to noise ratio. This has traditionally been done by visually inspecting the amount of overlap in the spectral regions associated with a certain analyte and performing quantification using the peak(s) that appear to be the least affected by such problems[17–19]. For a fully automated quantification method however, the peak selection has to be performed by a computer algorithm rather than humans.

Recently, manual targeted spectral profiling assisted by software such as *Chenomx NMRSuite* has become popular[20, 21]; this has made quantification of many metabolites in complex biological mixtures using ^1H -NMR possible[22, 23]. Performing targeted spectral profiling is however quite labour-intensive and often additional experiments have to be performed to aid the assignment of peaks in crowded regions of the spectra[22].

In this work, we present a complete automated pipeline of methods for peak detection, alignment, peak selection, background correction and quantification. The peak detection and alignment procedures have previously been described[5, 24] while the peak selection, background correction and quantification methods have not. Using this pipeline, we can go from raw spectra to tables of absolute concentrations with little or no user input. We present validation of the pipeline using synthetic metabolite mixtures with known concentrations. We also validate by comparing quantification of metabolites using our pipeline on urine samples with targeted spectral profiling using *Chenomx NMRSuite 7.1* (Chenomx Inc. Edmonton, Canada). To illustrate the usefulness of the pipeline, we apply it to a toxicology study on rat urine and show that the metabolite concentrations determined by the pipeline can be interpreted in terms of the lesions developed by the animals.

Experimental

Dataset A

Synthetic Arabidopsis mixture preparation—Twenty-four synthetic mixtures were prepared for this experiment. Each sample contained twenty-six metabolites present in *Arabidopsis thaliana* tissue extracts and three non-biological standards (HEPES, Sorbitol and DSS). All mixtures were prepared from pure standards (Sigma) suspended in 99.9% D_2O with 50 mM phosphate buffer, 500 μM sodium azide and 500 μM DSS. Samples were

titrated to an observed pH of 7.400 ($\pm$ 0.004). Each mixture contained nineteen compounds with invariant concentrations (all 5 mM including HEPES and Sorbitol) and seven metabolites with variable concentrations ranging from 5.5-29.1 mM. Although each mixture had a unique metabolite profile, the samples were designed to group into six classes with biologically relevant concentrations and standard deviations. The concentrations are shown in Table 7 in the supplementary information. The synthetic mixture samples are further discussed in the original publication[25].

NMR spectroscopy—NMR spectra were acquired on a Varian 600 MHz spectrometer (Varian Inc., Palo Alto, CA, USA) equipped with a 5 mm triple resonance cold probe and a sample changer. The probe was tuned, matched, and shimmed by hand for the first sample. All subsequent samples were collected at using an automated shimming and data acquisition macro written in-house. 1D ^1H -NMR spectra of each sample were collected using 90° pulses (9.625 μs) with four acquisitions, four silent scans, and a pre-acquisition delay of 1 second and an acquisition time of 2 seconds. Spectral acquisition parameters used were: 4 FIDs were collected into 32k complex data points, 10000 Hz spectral width, at 298 K. The data was auto-phased and baseline corrected using in-house developed software.

Dataset B

Sample and batches—Dataset B consists of 20 ^1H -NMR spectra measured on rat urine. In the study, rats were dosed with tetracycline once per day according to Table 1. Urine samples were collected for 17 hours during day 3, 7 hours after dosing. The urine samples were stored at -80°C and were thawed at room temperature before analysis. 400 μl urine was mixed with 200 μl 0.2 M phosphate buffer (pH 7.4) in 1.5 ml Eppendorf tubes and was centrifuged at 6350G and 4°C for 5 minutes. 500 μl of the supernatant was transferred to 5 mm NMR-tubes to which 50 μl TSP solution in D_2O (1 mg TSP/ml D_2O) was added. The tubes were ultrasonicated and placed in the NMR spectrometer. From each urine sample, 80 μl was pooled group-wise to obtain a total of 400 μl per group. These pooled samples were then treated according to the procedure described above. This results in 18 samples (5 for each group plus one pooled sample per group), two samples were run in duplicates yielding a total of 20 spectra. The animal study and the treatment of the study data were performed according to national study regulations.

NMR data acquisition—NMR data were acquired using a Bruker AV600 spectrometer at 600.23 MHz for ^1H observation (Bruker BioSpin, Rheinstetten, Germany). A 5 mm BBI probe was used for the study. Spectra were acquired using a standard Bruker NOESY presaturation pulse program (relaxation delay– 90° – τ_1 – 90° – τ_m – 90° –acquire-FID), where τ_m = 100 ms. The residual water resonances were suppressed by presaturation during the relaxation and mixing time (τ_m). Spectral acquisition parameters used were as follows: 512 FIDs were collected into 64k complex data points, 12019 Hz spectral width, 2.73 s acquisition time, 4.83 s total pulse recycle delay, at 300 K.

NMR spectral data processing—The spectra were corrected for phase and baseline distortions, and referenced internally to the TSP signal at 0 ppm using in-house automatic

software (*PhaseCore ver. 4.0*, by Dr. Ralf Torgrip) for Matlab. The spectra were normalized using the intensity of the TSP signal.

Dataset C

Samples and batches—The rat urine ethionine dataset has been described in earlier paper[26]. Briefly; urine was sampled twice a day (at 0-7 h and 8-24 h) on five and two days before, and days 1-7 after, the onset of ethionine dosing. The samples were stored on ice during collection, and then 1% (w/v) sodium azide was added to 1 mL portions, which were centrifuged at 500G for 10 min. The resulting supernates were stored at -80°C . After thawing, portions of the samples (400 μL) were mixed with 200 μL of 0.2 M phosphate buffer ($\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH = 7.4), and TSP (3-trimethylsilyl-1-[2,2,3,3,- $^2\text{H}_4$] propionate, internal standard) prepared in deuterium oxide (D_2O) was added to a final concentration of 0.09 mg/mL. The specifics for each group can be found in Table 2. The animal study and the treatment of the study data were performed according to national study regulations. The study has been presented before in several publications[5, 24, 26].

NMR data acquisition—NMR data were acquired using a Bruker DRX-600 spectrometer operating at 600.23 MHz for ^1H observation (Bruker Analytische Messtechnik GmbH, Rheinstetten, Germany). A 4 mm FISEI ^1H - ^{13}C Z-GRD probe was used for the study. Spectra were acquired using a standard Bruker NOESY presaturation pulse program (relaxation delay- 90° - τ_1 - 90° - τ_m - 90° -acquire-FID), where $\tau_m = 100$ ms. The residual water resonances were suppressed by presaturation during the relaxation and mixing time (τ_m). Spectral acquisition parameters used were as follows: 64 FIDs were collected into 32k complex data points, 12019 Hz spectral width, 2.73 s acquisition time, 4.83 s total pulse recycle delay, at 300 K.

NMR spectral data processing—The acquired FIDs were zero filled to double the number of data points and multiplied by an exponential line broadening function of 0.3 Hz prior to Fourier transformation. The spectra were corrected for phase and baseline distortions, and referenced internally to the TSP signal at 0 ppm using in-house automatic software (*PhaseCore ver. 4.0*, by Dr. Ralf Torgrip) for Matlab. The spectra were normalized using the intensity of the TSP signal.

Data Analysis

The general workflow of the pipeline for automatic quantification of metabolites in metabolomic ^1H -NMR datasets is presented in Figure 1. The spectra to be analyzed are peak-detected and then compared to a reference spectra with assigned chemical shifts and known metabolite concentrations (metabolite database). Generalized Fuzzy Hough Transform (GFHT) alignment is used to ensure that peaks have correct correspondence over the entire dataset. The intensity information for all peaks assigned to each metabolite is stored in (samples $\times$ peaks) tables. Automatic peak selection is performed for each metabolite to ensure that peaks with minimal overlap and correct correspondence are given higher priority in the quantification step. Quantification is performed using a single-point

Principal Component Regression (PCR)[27] model. The details regarding each step in the pipeline are described below.

Metabolite database

The presented pipeline for automated annotation and quantification of metabolites in ^1H -NMR metabolomic data requires a database containing peak positions in a single spectrum (henceforth referred to as the *reference spectrum*). Building such a database requires expertise in NMR-spectroscopy but can, once finished, be applied to any similar ^1H -NMR dataset. For datasets B and C, we used a database containing 20 metabolites, totaling 93 assigned peaks in the reference spectrum. The database was built using *Chenomx NMRSuite 7.1*. The metabolites can be found in Table 5. For validation and for demonstrating how a new metabolite database can be constructed without the use of *Chenomx*, we also built a new database for dataset A from scratch using correlation analysis on GFHT-aligned peak tables. The database can be found in Table 3.

Peak detection

Before annotation of the metabolites contained in the database, the spectra have to be peak detected. In this work, a zero-area filter[28] based method described in [24] is used. It uses the TSP peak shape as the basis for the zero-area filter instead of using a theoretical shape like a laurentzian. The method does not require any parameters except for a minimum signal to noise factor which was set to 3. For further details, refer to Ref[24].

GFHT alignment

The alignment method used in this work is based on earlier publications[24, 29] but has been improved in terms of reduced computational time and eliminated need for user input. Each peak in the metabolite database is aligned by maximizing the GFHT target function h in Equations 1 and 2. Note that the target function has been slightly altered from its original form[30] to increase speed of computation and to avoid excessive values of h in crowded regions of the spectra. This version is equivalent to using the maximum of the fuzzy region in the GFHT instead of using the sum.

$$\mathbf{s} = \mathbf{M}\boldsymbol{\alpha} + \mathbf{k} \quad (1)$$

$$h = \frac{\sum_{i=1}^N \exp\left(-(\mathbf{s}_i - \mathbf{z}_i)^2\right)}{N} \quad (2)$$

where $\mathbf{z}_i$ is the location of the detected peak closest to $\mathbf{s}_i$ in spectrum i (both measured in data points), N is the number of spectra (including the reference spectrum), h is the target function to be maximized by the algorithm, $\mathbf{M}$ is the shift model matrix ($N \times r$), where r is the number of selected *model peaks*, k is the position of the peak in the reference spectrum and

α is the set of independent Hough parameters. The entire algorithm can be found in the supplementary information.

Peak selection

For each metabolite in the database, a subset of the peaks is selected for quantification. This is performed by the following algorithm.

- The intensities of all the aligned peaks for the metabolite are put into a (samples $\times$ peaks) matrix $\mathbf{X}$.
- $\mathbf{X}$ is centered and scaled to unit standard deviation.
- PCA is performed; the peak with the highest absolute PC 2 loading value is removed from $\mathbf{X}$.
- The procedure is repeated until the explained variance of cross-validation reaches a pre-determined cut-off (set by user to 95-99% depending on the data complexity and noise level).
- If only 2 peaks remain and the explained variance still has not reached the cut-off, the peak with the highest relative standard deviation is removed and the remaining peak is used for quantification.

The method is shown for asparagine (dataset A) in Figures 2 and 3, 4 peaks are found to be outliers because of overlap or misalignments and are automatically removed before quantification. A cut-off of 99% explained variance was used for dataset A, 95% was used for datasets B and C. This was decided because the matrix is more complex in the urine samples and the metabolite concentration levels are lower.

Quantification

The selected peaks for the metabolite are used in a single-point calibration model similar to PCR. The reason for using only a single-point calibration model is that it requires only one reference concentration; an advantage because determining reference concentrations is labor-intensive.

- PCA is performed on the selected peaks for the metabolite, the PC 1 scores t_1 are used as the independent variable in the calibration model.
- The slope β of the single-point calibration model is calculated as

$$\beta = c_{\text{ref}} / t_{1\text{ref}}$$

The metabolite concentrations c of all the samples in the dataset is calculated using:

$$c = t_1 \beta$$

Results

Construction of a metabolite database for dataset A

Dataset A comprises 24 ^1H -NMR spectra of synthetic mixtures of metabolites created as a tool for error analysis of quantification methods[25]. They contain a mixture of 26 different small molecules including 7 components with variable concentrations, ranging from 5.5 mM to 29.1 mM. The objective of the database construction step is to find several peaks that originate from these metabolites and assign them an accurate chemical shift in one spectrum in the dataset. The intensities of these peaks are then used to calculate concentrations for the individual metabolites.

First, the dataset was peak-detected and aligned using the GFHT algorithm. This yielded 328 aligned peaks with correspondence over all the 24 samples. The first spectrum in the dataset was arbitrarily chosen as the reference spectrum for the metabolite database. For each of the 7 metabolites, one peak was assigned manually in the reference spectrum (assisted by ^1H - ^{13}C HSQC data, published by Lewis et al.[25]).

Correlations between the intensities of the assigned peak and all aligned peaks in the database were calculated. All peaks with R^2 values larger than 0.9 (Table 3) were compared to peaks in a pure metabolite spectrum (*Chenomx NMRSuite 7.1*) and included in the database for that metabolite if a close match was found. All peaks in the pure metabolite spectra could not be found in the aligned data. This is to be expected as some peaks in complicated multiplets are very small and some peaks may be completely or partially overlapped with other peaks.

Quantification of metabolites in dataset A

The method was applied to dataset A (synthetic Arabidopsis plant extract samples), the quantification results can be found in Table 8 in the appendix. Table 4 and Figure 4 show a comparison between our results and the true concentrations. Generally the results are in agreement, with an average R^2 value of 0.98 and average mean square relative error of prediction equal to 6.4%. Previously, an average error of 16.2% has been reported on the same data using manual integration of selected peaks and external standard calibration[25].

Construction of a metabolite database for dataset B and C

The metabolite database used for datasets B and C was built using *Chenomx NMRSuite 7.1*. For each metabolite, all library peak clusters found in Chenomx were compared manually to the reference spectrum. Peak clusters for which a close match was found in the Chenomx database were included in the peak list for that metabolite. The metabolite concentration obtained from Chenomx was used as the reference concentration in the metabolite database. This procedure generated between 1 and 16 peaks per metabolite (Table 5).

Quantification of metabolites in dataset B

The method was applied to dataset B (tetracycline rat urine dataset), the quantification results can be found in Table 9 in the appendix. As a reference, manual spectral profiling of the metabolites in the database (Table 5) was performed using *Chenomx NMRSuite 7.1*. A

comparison between the two methods can be found in Table 6 and Figure 5. For most of the metabolites the two methods were in agreement. The metabolites with the highest differences (maltose, betaine, allantoin) had their peaks in very crowded regions of the spectra, making manual spectral profiling of them difficult and somewhat arbitrary. 800 unassigned peaks with correspondence over the entire dataset were also generated by the GFHT-alignment algorithm; these peaks are not analyzed in this work.

Quantification of metabolites in dataset C

The method was applied to the 335 samples in dataset C (ethionine rat urine dataset); the quantification results can be found in Table 10 in the appendix. No validation against a second method was used for this dataset as it was deemed too time-consuming to perform manual spectral profiling of all 335 samples and the method has already been validated using datasets A and B.

Several metabolites were found to correlate with the conditions of the rats in the various stages of the study. Figures 6 and 7 show the concentrations of 15 metabolites for each animal group at each time-point. Concentrations of citrate, 2-oxoglutarate and succinate decreased rapidly during day 1 in the dosed group except the low-dose group (group 2). Group 2 has a slight decrease in succinate levels compared to group 1 from day 2 and onward ($p=0.003$, fold change=0.8). Group 4 returned to normal concentrations of those metabolites around day 5 (day 4 for succinate). The concentrations of glucose and alanine increased drastically for group 4 during day 3, the levels started to decrease again during day 6 but never reached normal levels. A similar pattern can be seen for methylsuccinate, but here the increase in concentrations can be seen already during day 2. Also, group 2 has an elevated level of methylsuccinate in the day-urine (marked as *pm* in Figures 6 and 7) from day 3 and onward ($p=6 \cdot 10^{-7}$, fold change=1.45) though much less so than group 4. N,N-dimethylglycine concentrations increase drastically in group 4 at day 5 and then decreases but never returns to normal levels. Trigonelline concentrations spikes during day 3 for groups 3 and 4, and then returns to normal levels for group 4 (group 3 was euthanized at this point). For the other metabolites, no clear pattern can be seen.

These patterns can also be seen in the metabolic trajectories in Figures 8 and 9. Especially when every concentration is scaled to unit standard deviation (Figure 9), it becomes clear which metabolite that is associated with each stage of the study for the high- and medium-dose groups. The low-dose group (group 2) cannot clearly be distinguished from the control groups (group 1 and 5) in these figures, indicating that the effect on their metabolism from the dosing is not very significant.

In addition to the peaks assigned to a particular metabolite, 743 unassigned peaks with correspondence over the entire dataset were aligned by the GFHT-algorithm; for an analysis of these peaks, refer to a previous publication on this dataset[5].

Conclusions

The pipeline for automatic annotation and quantification of metabolites presented in this work represents a significant improvement in high-throughput data analysis of ^1H -NMR data

of biological origin. Metabolite concentrations can be determined even in very difficult sample matrices such as urine where other automated methods have failed[15]. The level of automation in each step is very high. Only a few parameters have to be set by the user for each dataset; two parameters for the alignment method and one for the peak selection.

The method has been validated both against true concentrations in synthetic mixtures and against manual targeted spectral profiling using *Chenomx NMRSuite 7.1*. This enables us to compare our results with results from studies using Chenomx and also indicates that the concentrations obtained are in agreement with the actual concentrations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix

GFHT algorithm

The objective of the GFHT alignment algorithm is to maximize h in Equation 2 for each peak in the reference spectrum.

$$\mathbf{s} = \mathbf{M}\boldsymbol{\alpha} + \mathbf{k} \quad (1)$$

$$h = \frac{\sum_{i=1}^N \exp\left(-(\mathbf{s}_i - \mathbf{z}_i)^2\right)}{N} \quad (2)$$

where $\mathbf{z}_i$ is the location of the detected peak closest to $\mathbf{s}_i$ in spectrum i (both measured in data points), N is the number of spectra (including the reference spectrum), h is the target function to be maximized by the algorithm, $\mathbf{M}$ is the shift model matrix ($N \times r$), where r is the number of selected *model peaks*, $\mathbf{k}$ is the position of the peak in the reference spectrum and $\boldsymbol{\alpha}$ is the set of independent Hough parameters.

Definitions

$\mathbf{X}$ – The raw data matrix ($N \times m$), where N is the number of spectra and m is the number of data points per spectrum.

$\mathbf{A}$ – The aligned data matrix ($N \times p$), where p is the number of successfully aligned peaks.

MAX_MATCH – The maximum number of data points of “slack” the algorithm has when assigning a peak.

H_CUTOFF – The minimum value of h that is required to regard an alignment as successful.

Algorithm:

- 1 Define MAX_MATCH and H_CUTOFF. The actual values used can be found in the results section. We recommend values of 0-3 data points for MAX_MATCH and a value of 0.5-0.6 for H_CUTOFF.
- 2 Select a few (5-10) easily assignable peaks as model peaks. This is most easily done by finding local maxima in small segments of the spectra where one peak is known to dominate. Arrange the positions of each selected peak as columns in the shift model matrix $\mathbf{M}$. Subtract the position of the peak in the reference spectrum for each column from all values in that column (every column will have a zero at the reference spectrum position).
- 3 For every peak in the annotation database, perform the following steps:
 - a. In the peak detected reference spectrum, find the peak closest to the entry in the database. Set k equal to its position on the ppm axis.
 - b. Generate a random starting guess for $\mathbf{a}$ (which should have as many entries as $\mathbf{M}$ has columns).
 - c. Maximize the target function h in Equation 2 as a function of $\mathbf{a}$ using simplex optimization. Repeat step 3b to 3c several times with higher starting guesses if the optimization does not converge.
 - d. Adjust $\mathbf{s}$ slightly to match the closest detected peak if it is within MAX_MATCH data points. Do not recalculate h in this step.
 - e. If $h > H_CUTOFF$ add $\mathbf{s}$ as a new column in the peak location matrix $\mathbf{S}$, else disregard it. If $h < H_CUTOFF$ and the current peak is the only known peak for a metabolite, retry steps 3b to 3c again before giving up on the metabolite.
- 4 For every entry (i, j) in $\mathbf{S}$, perform the following steps:
 - a. Calculate a baseline value b equal to the minimum value in row i of $\mathbf{X}$ between the detected peak to the left of $\mathbf{S}(i, j)$ and the one to the right.
 - b. Assign an intensity for the peak in the aligned data matrix:

$$\mathbf{A}(i, j) = \mathbf{X}(\mathbf{I}, \mathbf{S}(i, j)) - b$$

Table 7

Reference concentrations of seven variable target metabolites in dataset A (mM).^a

Sample	Asparagine	Alanine	Glutamine	Proline	Valine	Sucrose	Malate
A1	9.76	5.52	15.74	15.12	11.46	7.21	9.76
A2	7.22	7.21	12.32	12.67	12.92	8.31	8.40
A3	9.36	6.89	11.89	15.32	9.33	6.11	9.77
A4	10.37	7.44	13.33	13.87	12.01	6.88	8.21
B1	21.79	10.17	8.60	10.36	22.68	16.07	29.05
B2	19.58	10.71	7.42	13.72	15.24	16.25	26.84
B3	17.80	14.31	10.74	11.35	17.33	20.41	23.51
B4	19.25	11.49	10.11	12.28	18.30	16.14	25.68
C1	15.32	19.32	16.26	27.21	15.97	20.48	14.20
C2	17.68	16.74	20.78	16.52	17.32	16.53	14.62

Sample	Asparagine	Alanine	Glutamine	Proline	Valine	Sucrose	Malate
C3	16.60	21.31	19.30	18.76	13.11	19.23	12.21
C4	18.00	18.80	18.90	25.44	12.25	21.08	12.58
D1	6.03	13.74	17.10	6.53	6.23	10.49	21.55
D2	6.81	14.42	15.61	6.87	6.54	13.50	17.94
D3	7.34	16.41	19.84	5.88	6.99	14.11	20.60
D4	5.97	14.31	15.32	7.50	7.66	14.63	21.33
E1	29.94	27.31	25.36	10.06	10.65	8.92	8.00
E2	23.24	22.48	28.92	8.43	11.53	8.74	5.90
E3	28.57	26.78	20.35	9.20	8.81	9.82	6.73
E4	22.95	22.67	25.43	9.78	8.32	9.76	6.36
F1	14.81	17.42	8.21	17.19	25.72	23.40	17.23
F2	12.17	15.87	5.78	20.55	20.11	26.51	19.03
F3	13.18	18.12	6.70	15.16	22.73	19.99	15.49
F4	13.75	18.00	6.72	19.19	26.31	23.49	16.32

² Each metabolite mixture contained 26 metabolites. Nineteen non-target metabolites were present at 5 mM and seven target metabolites were present with variable concentrations. Concentrations were chosen to segregate the samples into six groups (A through F) with biologically realistic means and standard deviations.

Table 8

Complete predicted concentrations in dataset A.

sample	Asparagine	Alanine	Glutamine	Proline	Valine	Sucrose	Malate
1	9,91	5,99	16,1	14,6	12,2	7,95	9,73
2	7,57	7,57	12,8	11,4	12,8	8,27	8,13
3	9,84	7,40	11,6	13,6	9,67	6,06	9,86
4	10,2	7,65	12,9	11,6	11,9	6,63	8,25
5	21,0	10,6	6,14	9,81	21,8	15,0	28,9
6	18,7	10,9	7,19	12,5	14,6	14,7	26,7
7	17,0	15,4	11,0	12,1	18,0	20,5	22,9
8	19,6	12,2	9,61	11,6	17,8	15,0	26,2
9	15,4	20,1	17,3	27,0	16,5	20,3	13,6
10	17,7	16,7	20,8	16,5	17,3	16,5	14,6
11	17,4	21,4	18,5	17,7	13,0	18,6	12,0
12	18,1	19,4	19,0	24,0	12,8	20,5	12,5
13	6,18	15,2	19,0	8,17	7,34	12,5	20,6
14	6,85	15,4	16,2	7,22	7,21	14,5	17,9
15	7,28	17,8	21,2	6,95	7,88	15,3	20,7
16	6,11	14,9	14,7	7,41	8,06	14,3	20,9
17	26,5	29,1	26,7	11,1	12,1	9,84	8,25
18	22,3	21,9	26,0	7,59	11,5	8,22	5,94

sample	Asparagine	Alanine	Glutamine	Proline	Valine	Sucrose	Malate
19	27,2	26,2	19,3	8,10	9,02	9,62	6,50
20	22,8	20,7	22,8	8,11	8,51	9,05	6,20
21	14,6	17,9	7,73	14,8	23,9	19,7	16,3
22	12,6	16,8	6,28	19,7	19,4	24,5	19,0
23	13,6	19,0	7,07	14,8	22,0	19,2	15,8
24	13,9	17,9	6,94	17,8	24,6	20,4	16,4

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Figure 1. Schematic workflow of the pipeline for automated annotation and quantification presented in this work.

Figure 2.

The PCA-based peak-selection method applied to asparagine (10 aligned peaks) in dataset A. As all peaks originate from one analyte, 1 PC should ideally describe 100% of the variability in the peak intensities. Peaks that do not cluster along PC 1 are removed before quantification.

Figure 3.

Explained variance of cross-validation from PCA models of three analytes from dataset A as a function of number of peaks removed. The curves for asparagine and malate have distinct knee-points which indicate the cut-off between removing low-quality peaks and removing high-quality peaks. The curve for proline does not have a distinct knee-point indicating that the peak quality is more evenly distributed among the peaks.

Figure 4.
Predicted vs. reference plot for the metabolites with variable concentration in dataset A.

Figure 5.
Predicted vs reference, sorted by ascending RMSEP.

Figure 6. Metabolite concentrations as a function of time for dataset C. The vertical bars indicate the within group standard deviation. Samplings marked with *am* indicate morning-urine (0-7 h) and samplings marked with *pm* indicate day-urine (8-24 h).

Figure 7.

Metabolite concentrations as a function of time for dataset C. The vertical bars indicate the within group standard deviation. Samplings marked with *am* indicate morning-urine (0-7 h) and samplings marked with *pm* indicate day-urine (8-24 h).

Figure 8.

The metabolic trajectories for the different groups in dataset C shown as a PCA bi-plot with no data pretreatment except mean-centering. The very early stage after dosing (day 1) can be seen as a slight shift down and right. The intermediate stage (day 2 to 3) is characterized by a shift left and up for the high-dose groups and the late stage (day 4 and onwards) by a shift down and left for the remaining high-dose group.

The metabolic trajectories for the different groups in dataset C shown as a PCA bi-plot after mean-centering and scaling to unit standard deviation. The intermediate stage of the study (day 1 pm to day 3) is characterized by a shift left and down for the high-dose groups and the late stage (day 4 and onwards) by a shift up and left for the remaining high-dose group.

Table 1

Animal groups and tetracycline dose.

Group	Number of animals	Dose levels (mg/kg/day)
G1	5	0
G3	5	250
G4	5	500

Table 2

Animal groups, ethionine dose and necroscopy.

Group	Number of animals	Dose levels (mg/kg/day)	Dosing period (days)	Necroscopy (day)
G1	5	0	7	8
G2	5	20	7	8
G3	5	80	7	8 [*]
G4	5	800	single dose	8
G5	5	0	single dose	2
G6	5	80	single dose	2
G7	5	800	single dose	2

* Group 3 was terminated after day 3 for ethical reasons.

Table 3

Metabolite database for dataset A

Analyte	Aligned peaks with $R^2 > 0.9$ and matching reference values (ppm in reference spectrum)	Assigned peaks
Asparagine	2.8304, 2.8434, 2.8586, 2.8716, 2.9253, 2.9324, 2.9535, 2.9607, 3.9932, 3.9990	10
Alanine	1.4610, 1.4730, 3.7881	3
Glutamine	2.1065, 2.1119, 2.1180, 2.1213, 2.1287, 2.1434, 2.1472, 2.1592, 2.1615, 2.4345, 2.4363, 2.4485, 2.4604, 2.4719, 2.4742, 2.4861	16
Proline	1.9653, 1.9678, 1.9785, 1.9902, 2.0022, 2.0136, 2.0650, 2.0778, 3.3149, 3.3342, 3.3461, 3.4199, 3.4319, 4.1179, 4.1217, 4.1324	16
Valine	0.9727, 0.9844, 1.0244, 1.0361, 2.2368, 2.2444, 2.2485, 2.2559, 2.2676, 3.6026	10
Sucrose	3.4439, 3.4604, 3.4762, 3.5360, 3.5423, 3.5528, 3.5589, 3.6652, 3.7354, 3.7515, 3.8019, 3.8072, 3.8118, 3.8204, 4.0260, 4.0405, 4.0548, 4.1980, 4.2125, 5.3987, 5.4051	21
Malate	4.2766, 4.2815, 4.2934, 4.2985	4

Table 4

Validation results for dataset A

Analyte	One-point calibration, reference vs. predicted.							
	Asparagine	Alanine	Glutamine	Proline	Valine	Sucrose	Malate	Average
slope	0.91	0.98	0.96	0.92	0.89	0.87	0.99	0.93
intercept	1.1	0.84	0.43	0.45	1.6	1.5	-0.009	0.85
R ²	0.99	0.98	0.97	0.97	0.99	0.96	0.99	0.98
RMSEP	0.87	0.87	1.2	1.2	0.80	1.3	0.38	0.87
RMSEP [%]	5.7	5.6	8.0	8.7	5.6	8.9	2.5	6.4

Table 5

Metabolites, with number of assigned peaks in the database. The database was built using Chenomx NMRSuite 7.1.

Glucose (16)	2-Oxoglutarate (6)	Betaine (2)	Maltose (3)
Creatinine (2)	Succinate (1)	Sucrose (9)	Mannose (2)
Taurine (6)	β -Alanine (2)	Trigonelline (8)	Allantoin (2)
Methylsuccinate (2)	Dimethylamine (1)	1-Methylnicotinamide (10)	3-Indoxylsulfate (16)
Alanine (2)	N,N-Dimethylglycine (2)	Xylose (2)	Citrate (4)

Table 6

Comparison with reference data from Chenomx NMRSuite 7.1.

	2-Oxoglutarate	N,N-Dimethyl- glycine	Succinate	Dimethyl-amine	Citrate	Glucose	Alanine
slope	0.98	1.0	1.0	0.97	0.98	1.1	0.97
intercept	-0.038	-0.006	-0.003	0.025	0.012	-0.11	0.014
R ²	1.00	0.99	0.998	0.99	0.99	0.98	0.94
RMSEP	0.19	0.011	0.028	0.017	0.36	0.10	0.013
Mean relative RMSEP [%]	3.1	3.1	4.19	4.4	4.4	4.4	8.1
	Trigonelline	Creatinine	Mannose	Cis-Aconitate	β -Alanine	Sucrose	Taurine
slope	0.97	0.94	1.1	1.06	0.97	1.2	1.04
intercept	-0.009	-0.036	-0.030	-0.006	0.007	-0.085	-0.15
R ²	0.97	0.97	0.96	0.93	0.90	0.95	0.91
RMSEP	0.019	0.28	0.025	0.053	0.018	0.065	0.58
Mean relative RMSEP [%]	8.7	9.0	9.5	10.2	11.7	13.1	14.0
	1-Methyl-nicotinamide	Methyl-succinate	3-Indoxyl-sulfate	Xylose	Maltose	Betaine	Allantoin
slope	1.2	0.65	1.3	1.06	0.32	1.9	0.55
intercept	-0.009	0.01	-0.033	0.034	3.1	-0.54	-1.8
R ²	0.99	0.57	0.98	0.59	0.16	0.80	0.42
RMSEP	0.014	0.011	0.091	0.11	1.7	0.66	6.0
Mean relative RMSEP [%]	20.8	25.3	27.2	29.0	52.6	59.0	78.0

Table 9

Complete predicted concentrations in dataset B.

sample	Glucose	Creatinine	Taurine	Methylsuccinate	Alanine	2-oxoglutarate	Succinate	beta-alanine	dimethylamine	N,N-Dimethylglycine	cis-Aconitate	Betaine	Sucrose	Trigonelline	1-Methylhydantoinamide	Xylose	Maltose	Mannose	Allantoin	3-Indoxylsulfate	Citrate	Leucine
1	1.43	2.06	4.22	0.0221	0.0768	1.78	0.254	0.0387	0.255	0.407	0.304	0.562	0.222	0.159	0.0622	0.268	2.02	0.132	9.37	0.0248	2.48	0.0112
2	1.98	2.79	3.45	0.0246	0.0913	4.64	0.654	0.0520	0.361	0.209	0.409	0.343	0.335	0.212	0.0410	0.179	2.93	0.158	10.1	0.123	4.34	0.0162
3	1.22	1.23	0.505	0.0126	0.0660	2.28	0.213	0.09909	0.179	0.167	0.257	0.227	0.120	0.0399	0.02385	0.0788	1.84	0.0774	6.53	0.0877	2.88	0.0132
4	1.78	1.98	1.57	0.0180	0.0771	6.77	0.699	0.0314	0.300	0.252	0.464	0.314	0.237	0.142	0.0183	0.162	2.54	0.150	10.9	0.313	7.10	0.0127
5	1.48	2.38	2.13	0.0185	0.0677	5.41	0.419	0.0312	0.305	0.300	0.431	0.336	0.228	0.158	0.0321	0.136	2.19	0.152	9.65	0.132	4.84	0.0123
6	1.06	1.90	2.94	0.0160	0.0648	2.23	0.280	0.0286	0.263	0.216	0.293	0.444	0.163	0.145	0.0380	0.135	1.52	0.0958	8.59	0.0628	2.18	0.00808
7	1.27	1.89	3.30	0.0208	0.0679	2.36	0.371	0.0337	0.254	0.246	0.302	0.376	0.197	0.138	0.0433	0.150	1.87	0.118	8.26	0.0679	2.32	0.0100
8	0.463	0.532	0.946	0.00551	0.0206	0.553	0.123	0.00771	0.0751	0.0473	0.0836	0.0410	0.0400	0.0271	0.00907	0.0390	0.689	0.0434	2.88	0.0305	0.630	0.00162
9	1.65	2.89	2.89	0.0241	0.118	8.53	0.885	0.0377	0.353	0.245	0.623	0.422	0.305	0.220	0.0439	0.281	2.26	0.287	17.6	0.395	11.1	0.0154
10	2.71	2.42	2.28	0.0186	0.0912	11.47	1.09	0.0623	0.350	0.279	0.603	0.287	0.255	0.201	0.0172	0.155	3.78	0.210	13.0	0.334	11.9	0.0271
11	1.61	1.89	4.07	0.0222	0.0758	2.07	0.391	0.0364	0.254	0.401	0.299	0.524	0.220	0.150	0.0565	0.261	2.33	0.109	8.57	0.0225	2.36	0.0123
12	1.23	2.42	4.24	0.0183	0.0876	3.12	0.393	0.0398	0.283	0.339	0.318	0.664	0.218	0.186	0.0312	0.144	1.74	0.129	13.1	0.128	5.08	0.0151
13	1.79	2.26	4.23	0.0188	0.0841	3.56	0.574	0.0343	0.285	0.316	0.347	0.651	0.228	0.188	0.0314	0.143	2.47	0.105	12.8	0.119	5.09	0.0191
14	1.54	2.35	4.32	0.0325	0.0867	5.57	0.431	0.0486	0.348	0.336	0.487	0.220	0.263	0.204	0.0336	0.171	2.24	0.145	9.81	0.144	5.36	0.0184
15	1.67	2.60	4.74	0.0268	0.0984	3.06	0.693	0.0451	0.283	0.369	0.642	0.653	0.571	0.232	0.315	0.152	2.27	0.223	17.6	0.462	5.81	0.0174
16	1.31	2.24	1.48	0.0156	0.0652	4.75	0.667	0.0257	0.295	0.187	0.408	0.206	0.184	0.176	0.0106	0.126	1.84	0.185	12.7	0.546	6.19	0.0107
17	1.73	2.78	3.57	0.0251	0.0942	5.62	0.601	0.0491	0.322	0.301	0.503	0.390	0.358	0.219	0.102	0.183	2.45	0.190	14.1	0.264	6.76	0.0131
18	1.74	3.31	5.34	0.0380	0.0942	3.57	0.428	0.0566	0.409	0.273	0.434	0.285	0.374	0.246	0.0877	0.204	2.51	0.227	9.53	0.106	2.80	0.0179
19	1.47	2.18	0.894	0.0204	0.0811	8.84	0.658	0.0400	0.288	0.439	0.543	0.642	0.329	0.181	0.0176	0.236	2.02	0.151	12.3	0.353	8.85	0.0180
20	1.94	2.21	2.59	0.0209	0.0853	6.10	0.254	0.0346	0.359	0.178	0.540	0.225	0.327	0.177	0.0297	0.202	2.76	0.155	13.5	0.348	7.15	0.0232

Table 10

Complete predicted concentrations in dataset C.

Sampling time	Group	Rat number	Glucose	Creatinine	Methylsuccinate	Alanine	2-Oxoglutarate	Succinate	Dimethylamine	N,N-Dimethylglycine	Betaine	Sucrose	Trigonelline	Xylose	Allantoin	3-Indoxylsulfate	Citrate
day -5	1	1	0.230	1.322	0.0093	0.050	4.611	0.429	0.503	0.194	0.544	0.025	0.101	1.645	9.32	1.24	11.27
day -5	1	2	0.112	1.050	0.0064	0.027	2.391	0.338	0.115	0.105	0.159	0.041	0.071	0.527	6.98	1.07	6.11
day -5	1	3	0.128	0.963	0.0058	0.033	2.642	0.479	0.085	0.100	0.258	0.025	0.073	0.910	6.52	1.02	5.95
day -5	1	4	0.124	1.089	0.0127	0.028	2.901	0.263	0.069	0.145	0.351	0.046	0.114	0.037	8.04	1.06	8.57
day -5	1	5	0.143	0.906	0.0072	0.031	2.586	0.237	0.122	0.076	0.170	0.047	0.070	0.170	5.93	1.18	6.55
day -5	2	6	0.120	1.155	0.0010	0.045	2.381	0.167	0.243	0.070	0.259	0.027	0.061	1.236	5.92	0.61	6.05
day -5	2	7	0.105	0.724	0.0041	0.026	2.320	0.196	0.115	0.064	0.190	0.034	0.048	1.272	5.45	0.93	4.85
day -5	2	8	0.143	0.983	0.0074	0.033	2.794	0.265	0.200	0.061	0.256	0.047	0.082	0.560	7.64	0.75	6.61
day -5	2	9	0.078	0.529	0.0027	0.017	1.955	0.141	0.092	0.040	0.106	0.022	0.048	0.089	4.43	0.62	4.44
day -5	2	10	0.146	0.941	0.0063	0.040	3.177	0.301	0.147	0.186	0.224	0.028	0.081	0.305	6.52	1.04	7.35
day -5	3	11	0.188	1.401	0.0068	0.040	6.518	0.457	0.235	0.176	0.265	0.067	0.102	0.788	8.95	1.53	11.29
day -5	3	12	0.169	1.305	0.0069	0.031	4.407	0.438	0.223	0.061	0.112	0.048	0.086	0.414	8.02	1.43	9.49
day -5	3	13	0.114	0.558	0.0044	0.026	2.038	0.242	0.089	0.044	0.096	0.029	0.050	0.112	4.61	0.70	4.80
day -5	3	14	0.200	1.287	0.0071	0.037	5.960	0.330	0.283	0.167	0.240	0.040	0.106	0.181	8.78	1.48	12.38
day -5	3	15	0.151	0.870	0.0051	0.036	3.673	0.267	0.201	0.100	0.252	0.026	0.078	0.784	6.65	1.07	7.92
day -5	4	16	0.161	1.145	0.0075	0.033	5.758	0.618	0.202	0.085	0.258	0.055	0.086	0.092	6.75	1.36	9.48
day -5	4	17	0.139	0.897	0.0028	0.031	3.961	0.321	0.176	0.131	0.241	0.043	0.071	0.058	5.42	1.13	7.46
day -5	4	18	0.091	0.773	0.0011	0.026	2.420	0.375	0.147	0.089	0.204	0.015	0.059	0.081	6.02	0.89	5.22
day -5	4	19	0.108	0.764	0.0052	0.020	1.455	0.267	0.126	0.067	0.147	0.024	0.055	0.236	5.72	0.92	4.15
day -5	4	20	0.125	0.948	0.0055	0.025	1.791	0.406	0.145	0.056	0.267	0.035	0.066	0.305	5.92	1.05	3.99
day -5	5	21	0.100	0.633	0.0031	0.016	2.382	0.167	0.106	0.080	0.161	0.019	0.045	0.460	4.68	0.77	4.74
day -5	5	22	0.147	1.213	0.0100	0.040	2.678	0.248	0.140	0.159	0.155	0.040	0.082	0.104	7.18	1.56	6.06
day -5	5	23	0.158	1.049	0.0079	0.056	4.098	0.328	0.193	0.096	0.263	0.017	0.075	0.708	7.00	0.64	8.27
day -5	5	24	0.089	0.930	0.0072	0.023	1.848	0.215	0.125	0.105	0.173	0.032	0.066	0.811	6.86	1.07	4.64
day -5	5	25	0.100	0.743	0.0017	0.026	2.760	0.384	0.125	0.068	0.291	0.018	0.062	0.330	5.09	0.89	6.33
day -5	6	26	0.097	1.040	0.0069	0.020	2.609	0.245	0.129	0.086	0.183	0.039	0.065	0.268	6.48	1.36	5.33
day -5	6	27	0.071	0.639	0.0006	0.025	0.068	0.066	0.100	0.017	0.110	0.011	0.018	0.135	4.05	0.07	0.25
day -5	6	28	0.136	0.913	0.0059	0.030	3.688	0.267	0.208	0.127	0.295	0.025	0.085	0.525	5.82	1.22	7.80

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Sampling time	Group	Rat number	Glucose	Creatinine	Methylsuccinate	Alanine	2-Oxoglutarate	Succinate	Dimethylamine	N,N-Dimethylglycine	Betaine	Sucrose	Trigonelline	Xylose	Allantoin	3-Indoxylsulfate	Citrate
day -5	6	29	0.118	1.078	0.0026	0.026	2.420	0.292	0.158	0.070	0.222	0.025	0.077	0.043	6.45	0.80	4.98
day -5	6	30	0.196	1.118	0.0059	0.036	3.055	0.209	0.201	0.159	0.335	0.037	0.077	0.996	7.05	1.26	7.47
day -5	7	31	0.166	1.197	0.0073	0.035	2.643	0.320	0.146	0.094	0.341	0.035	0.075	0.181	6.39	1.05	6.04
day -5	7	32	0.163	1.194	0.0068	0.037	3.084	0.510	0.212	0.065	0.307	0.053	0.081	0.090	8.14	1.14	7.18
day -5	7	33	0.077	0.658	0.0047	0.019	2.115	0.142	0.103	0.048	0.144	0.021	0.040	1.110	4.90	0.65	4.47
day -5	7	34	0.106	1.085	0.0055	0.047	2.838	0.215	0.229	0.064	0.245	0.024	0.066	0.272	5.53	0.64	5.66
day -5	7	35	0.134	0.984	0.0069	0.031	4.208	0.347	0.202	0.119	0.151	0.045	0.078	0.751	7.43	1.22	8.28
day -2	1	1	0.276	1.404	0.0186	0.032	6.573	0.443	0.382	0.155	0.053	0.048	0.106	0.837	8.86	0.70	11.64
day -2	1	2	0.156	1.016	0.0090	0.014	2.972	0.432	0.125	0.102	0.006	0.007	0.063	0.045	6.68	0.51	5.89
day -2	1	3	0.226	1.199	0.0126	0.029	2.536	0.554	0.240	0.124	0.058	0.026	0.076	1.823	6.27	0.80	5.38
day -2	1	4	0.181	0.971	0.0131	0.016	4.605	0.394	0.197	0.108	0.016	0.024	0.064	1.117	6.13	0.59	9.16
day -2	1	5	0.235	1.354	0.0155	0.032	2.794	0.260	0.198	0.071	0.001	0.027	0.069	0.063	7.94	0.75	6.58
day -2	2	6	0.253	1.298	0.0288	0.024	4.196	0.303	0.954	0.114	0.077	0.034	0.093	1.217	7.25	0.73	9.09
day -2	2	7	0.164	1.015	0.0110	0.020	3.619	0.448	0.189	0.045	0.027	0.017	0.051	0.807	5.98	0.55	6.74
day -2	2	8	0.239	1.103	0.0144	0.030	4.293	0.294	0.241	0.063	0.017	0.026	0.091	0.066	7.32	0.78	9.60
day -2	2	9	0.129	0.675	0.0073	0.015	2.802	0.181	0.126	0.053	0.021	0.020	0.051	0.568	4.74	0.52	5.74
day -2	2	10	0.289	1.346	0.0172	0.042	5.637	0.326	0.249	0.286	0.033	0.040	0.101	0.313	9.33	0.96	11.92
day -2	3	11	0.247	1.469	0.0148	0.024	4.586	0.522	0.310	0.169	0.029	0.031	0.094	0.496	8.77	0.92	11.59
day -2	3	12	0.265	1.585	0.0136	0.026	6.700	0.450	0.279	0.082	0.006	0.033	0.110	0.089	10.35	1.08	10.84
day -2	3	13	0.161	0.761	0.0102	0.023	2.701	0.290	0.129	0.050	0.033	0.022	0.059	0.175	5.46	0.51	6.24
day -2	3	14	0.294	1.515	0.0130	0.023	7.213	0.298	0.326	0.119	0.024	0.031	0.083	0.363	7.87	1.04	13.73
day -2	3	15	0.159	0.904	0.0122	0.022	2.262	0.177	0.158	0.063	0.020	0.024	0.056	0.645	5.98	0.69	5.60
day -2	4	16	0.149	0.815	0.0118	0.013	3.122	0.430	0.132	0.066	0.011	0.017	0.049	0.052	5.58	0.46	6.53
day -2	4	17	0.201	1.009	0.0147	0.021	5.172	0.419	0.208	0.127	0.030	0.021	0.066	0.328	6.49	0.66	9.50
day -2	4	18	0.220	1.270	0.0128	0.024	5.661	0.346	0.261	0.085	0.013	0.031	0.078	0.615	7.99	0.77	8.73
day -2	4	19	0.178	0.959	0.0090	0.026	2.595	0.325	0.190	0.074	0.024	0.007	0.078	0.077	7.12	0.79	5.91
day -2	4	20	0.167	1.063	0.0156	0.018	2.627	0.298	0.166	0.050	0.027	0.019	0.059	0.125	5.68	0.48	5.33
day -2	5	21	0.220	1.125	0.0151	0.034	3.714	0.333	0.209	0.124	0.017	0.032	0.079	1.026	7.51	0.81	7.78
day -2	5	22	0.406	1.623	0.0261	0.042	4.977	0.875	0.228	0.166	0.004	0.012	0.098	0.119	9.89	0.84	10.54
day -2	5	23	0.195	1.135	0.0097	0.025	4.647	0.279	0.250	0.090	0.025	0.030	0.071	1.078	6.26	0.83	9.41
day -2	5	24	0.132	1.122	0.0080	0.014	1.643	0.286	0.184	0.087	0.010	0.019	0.051	1.424	7.06	0.77	4.53

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Sampling time	Group	Rat number	Glucose	Creatinine	Methylsuccinate	Alanine	2-Oxoglutarate	Succinate	Dimethylamine	N,N-Dimethylglycine	Betaine	Sucrose	Trigonelline	Xylose	Allantoin	3-Indoxylsulfate	Citrate
day -2	5	25	0.157	0.985	0.0099	0.021	2.774	0.367	0.170	0.047	0.069	0.020	0.065	0.693	6.05	0.61	7.05
day -2	6	26	0.232	1.139	0.0146	0.016	5.002	0.526	0.228	0.138	0.009	0.028	0.094	0.648	8.10	0.72	8.95
day -2	6	27	0.311	1.583	0.0140	0.033	7.122	0.711	0.611	0.144	0.033	0.035	0.115	0.766	9.71	0.88	13.45
day -2	6	28	0.307	1.366	0.0191	0.026	4.157	0.417	0.243	0.115	0.049	0.027	0.081	0.351	7.95	0.88	8.78
day -2	6	29	0.186	1.493	0.0203	0.024	0.547	0.205	0.199	0.044	0.015	0.027	0.059	1.097	6.69	0.49	1.55
day -2	6	30	0.202	1.319	0.0175	0.025	3.136	0.201	0.220	0.139	0.021	0.029	0.067	0.636	7.97	0.79	8.91
day -2	7	31	0.252	1.479	0.0200	0.026	3.045	0.256	0.235	0.123	0.026	0.029	0.067	0.675	7.84	0.91	8.24
day -2	7	32	0.340	2.252	0.0252	0.032	5.863	0.510	0.443	0.094	0.061	0.045	0.115	0.476	11.15	1.20	12.60
day -2	7	33	0.123	1.001	0.0059	0.010	1.853	0.170	0.152	0.046	0.014	0.011	0.040	0.777	5.62	0.57	5.18
day -2	7	34	0.207	1.283	0.0093	0.020	4.993	0.509	0.257	0.160	0.039	0.026	0.096	0.196	8.17	1.14	11.34
day -2	7	35	0.377	2.126	0.0208	0.045	7.153	0.468	0.389	0.198	0.058	0.047	0.142	0.606	12.02	1.57	13.32
day 1 am	1	1	0.193	1.385	0.0209	0.055	6.147	0.389	0.241	0.195	0.163	0.860	0.102	0.608	8.16	0.75	10.49
day 1 am	1	2	0.144	1.394	0.0104	0.026	3.239	0.530	0.177	0.121	0.047	0.627	0.072	0.083	8.58	0.33	6.03
day 1 am	1	3	0.131	1.210	0.0118	0.031	3.007	0.488	0.176	0.114	0.045	0.526	0.078	0.791	6.54	0.65	7.01
day 1 am	1	4	0.102	0.814	0.0103	0.020	2.608	0.374	0.138	0.092	0.038	0.488	0.061	0.058	5.13	0.60	5.91
day 1 am	1	5	0.064	0.563	0.0100	0.021	1.545	0.139	0.075	0.032	0.013	0.205	0.023	0.097	2.65	0.15	2.75
day 1 am	2	6	0.076	0.612	0.0065	0.014	2.396	0.176	0.105	0.038	0.006	0.539	0.037	0.095	3.49	0.35	5.00
day 1 am	2	7	0.115	0.923	0.0093	0.027	3.038	0.344	0.158	0.060	0.025	0.643	0.056	0.666	4.89	0.46	5.83
day 1 am	2	8	0.198	1.155	0.0134	0.033	4.848	0.263	0.171	0.074	0.036	0.801	0.090	0.555	6.91	0.91	9.79
day 1 am	2	9	0.097	0.715	0.0064	0.020	3.230	0.207	0.118	0.040	0.011	0.339	0.048	0.109	4.45	0.47	5.36
day 1 am	2	10	0.216	1.262	0.0152	0.049	3.849	0.268	0.185	0.213	0.076	0.948	0.082	0.658	7.42	0.96	8.64
day 1 am	3	11	0.156	1.151	0.0180	0.032	3.956	0.554	0.152	0.120	0.098	0.446	0.091	0.061	7.71	0.75	9.59
day 1 am	3	12	0.183	1.182	0.0110	0.025	5.011	0.473	0.167	0.070	0.029	0.352	0.065	0.069	6.01	0.61	8.17
day 1 am	3	13	0.106	0.496	0.0063	0.022	2.501	0.227	0.077	0.050	0.022	0.350	0.044	0.114	3.53	0.21	5.32
day 1 am	3	14	0.178	1.183	0.0125	0.030	7.588	0.307	0.189	0.123	0.039	0.925	0.073	0.177	6.44	0.88	11.94
day 1 am	3	15	0.145	0.713	0.0116	0.029	3.480	0.316	0.103	0.081	0.035	0.357	0.058	0.605	4.67	0.45	6.53
day 1 am	4	16	0.142	0.790	0.0223	0.036	4.894	0.288	0.125	0.105	0.230	0.411	0.066	0.131	3.80	0.54	7.80
day 1 am	4	17	0.202	1.027	0.0366	0.049	5.493	0.337	0.194	0.247	0.573	0.685	0.080	0.107	5.26	0.76	11.07
day 1 am	4	18	0.129	0.793	0.0148	0.050	0.918	0.064	0.127	0.106	0.061	0.388	0.056	0.606	3.43	0.27	2.42
day 1 am	4	19	0.174	0.827	0.0040	0.024	1.931	0.113	0.105	0.094	0.054	0.364	0.061	0.218	4.41	0.53	4.90
day 1 am	4	20	0.120	0.712	0.0214	0.022	2.455	0.184	0.112	0.095	0.066	0.261	0.044	0.306	3.72	0.42	5.55

Sampling time	Group	Rat number	Glucose	Creatinine	Methylsuccinate	Alanine	2-Oxoglutarate	Succinate	Dimethylamine	N,N-Dimethylglycine	Betaine	Sucrose	Trigonelline	Xylose	Allantoin	3-Indoxylsulfate	Citrate
day 1 am	5	21	0.103	0.868	0.0099	0.023	2.035	0.229	0.126	0.066	0.013	0.819	0.044	0.355	4.82	0.48	3.95
day 1 am	5	22	0.135	0.959	0.0147	0.034	2.271	0.357	0.125	0.120	0.035	0.342	0.061	0.055	5.36	0.61	5.41
day 1 am	5	23	0.130	1.040	0.0084	0.035	4.888	0.339	0.206	0.082	0.026	0.707	0.058	0.482	5.60	0.66	8.51
day 1 am	5	24	0.098	0.877	0.0087	0.021	1.322	0.314	0.133	0.102	0.031	0.749	0.048	0.890	5.14	0.49	3.62
day 1 am	5	25	0.144	1.022	0.0108	0.029	3.106	0.413	0.152	0.058	0.025	0.608	0.078	0.473	6.31	0.67	8.26
day 1 am	6	26	0.069	0.503	0.0058	0.015	1.665	0.198	0.078	0.071	0.018	0.169	0.033	0.425	3.22	0.25	3.11
day 1 am	6	27	0.141	1.224	0.0094	0.035	3.505	0.366	0.193	0.096	0.037	0.390	0.069	0.245	5.63	0.62	7.86
day 1 am	6	28	0.084	1.186	0.0117	0.032	4.420	0.294	0.225	0.100	0.015	0.438	0.069	0.476	6.32	0.78	9.50
day 1 am	6	29	0.123	1.074	0.0050	0.027	1.430	0.203	0.131	0.085	0.031	0.279	0.071	0.086	5.91	0.43	3.39
day 1 am	6	30	0.157	1.246	0.0137	0.034	4.430	0.309	0.228	0.159	0.043	0.421	0.056	0.511	7.04	0.61	9.17
day 1 am	7	31	0.193	1.062	0.0387	0.041	2.658	0.163	0.159	0.224	0.442	0.592	0.075	0.257	4.51	0.73	7.11
day 1 am	7	32	0.163	0.861	0.0294	0.032	2.472	0.121	0.138	0.087	0.119	0.558	0.067	0.557	3.83	0.51	4.94
day 1 am	7	33	0.112	0.639	0.0172	0.030	2.417	0.157	0.103	0.094	0.299	0.443	0.044	0.461	2.93	0.39	5.51
day 1 am	7	34	0.134	0.650	0.0238	0.017	3.354	0.218	0.124	0.113	0.195	0.459	0.053	0.097	3.49	0.42	6.55
day 1 am	7	35	0.176	0.872	0.0239	0.029	3.711	0.182	0.152	0.151	0.218	1.073	0.059	0.103	3.97	0.69	7.44
day 1 pm	1	1	0.495	1.427	0.0269	0.050	7.669	0.526	0.192	0.248	0.335	0.850	0.141	0.453	10.04	0.54	10.06
day 1 pm	1	2	0.233	1.020	0.0097	0.032	4.565	0.561	0.144	0.193	0.167	0.784	0.095	0.229	6.19	0.39	6.52
day 1 pm	1	3	0.266	1.435	0.0124	0.035	4.831	0.730	0.189	0.219	0.333	0.968	0.141	0.392	8.89	0.58	7.80
day 1 pm	1	4	0.378	1.262	0.0207	0.041	5.554	0.533	0.169	0.269	0.267	1.285	0.134	0.401	7.56	0.76	7.98
day 1 pm	1	5	0.437	1.689	0.0274	0.057	5.913	0.488	0.184	0.160	0.283	0.824	0.132	0.405	9.19	0.53	9.15
day 1 pm	2	6	0.475	0.983	0.0176	0.031	6.962	0.433	0.133	0.161	0.231	0.648	0.124	0.489	6.87	0.43	9.71
day 1 pm	2	7	0.249	0.957	0.0134	0.031	4.579	0.593	0.135	0.127	0.188	0.663	0.099	0.280	6.29	0.37	5.71
day 1 pm	2	8	0.256	0.885	0.0154	0.029	4.675	0.301	0.149	0.090	0.207	0.511	0.093	0.270	6.21	0.34	7.86
day 1 pm	2	9	0.341	1.258	0.0139	0.032	6.553	0.323	0.204	0.098	0.370	0.718	0.104	0.303	7.81	0.53	8.87
day 1 pm	2	10	0.352	1.446	0.0226	0.060	6.223	0.409	0.185	0.306	0.269	0.874	0.112	0.379	8.66	0.55	10.62
day 1 pm	3	11	0.177	0.825	0.0105	0.019	1.784	0.193	0.103	0.079	0.133	0.200	0.040	0.096	4.52	0.16	3.30
day 1 pm	3	12	0.317	1.247	0.0150	0.026	4.118	0.423	0.168	0.062	0.280	0.823	0.066	0.165	5.62	0.49	4.64
day 1 pm	3	13	0.127	0.846	0.0077	0.024	2.072	0.168	0.102	0.035	0.089	0.319	0.028	0.093	4.38	0.19	3.13
day 1 pm	3	14	0.117	0.438	0.0045	0.013	0.994	0.118	0.069	0.043	0.058	0.199	0.018	0.067	2.13	0.12	1.32
day 1 pm	3	15	0.269	0.729	0.014	0.028	3.447	0.351	0.103	0.087	0.133	0.278	0.060	0.219	4.76	0.178	5.14
day 1 pm	4	16	0.085	0.418	0.017	0.015	0.480	0.102	0.020	0.069	0.053	0.254	0.011	0.068	1.52	0.136	0.87

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Sampling time	Group	Rat number	Glucose	Creatinine	Methylsuccinate	Alanine	2-Oxoglutarate	Succinate	Dimethylamine	N,N-Dimethylglycine	Betaine	Sucrose	Trigonelline	Xylose	Allantoin	3-Indoxylsulfate	Citrate
day 1 pm	4	17	0.462	1.136	0.037	0.046	1.651	0.151	0.139	0.190	0.045	0.676	0.033	0.235	4.32	0.345	2.09
day 1 pm	4	18	0.195	0.642	0.009	0.019	0.188	0.117	0.067	0.090	0.080	0.101	0.018	0.127	2.79	0.093	0.49
day 1 pm	4	19	0.198	0.634	0.015	0.021	0.698	0.068	0.077	0.076	0.057	0.547	0.019	0.150	2.88	0.305	1.37
day 1 pm	4	20	0.364	0.526	0.011	0.019	0.256	0.081	0.046	0.060	0.013	0.251	0.023	0.078	2.53	0.155	0.63
day 1 pm	5	21	0.340	0.861	0.016	0.030	3.452	0.365	0.137	0.175	0.130	0.629	0.086	0.278	5.63	0.369	4.64
day 1 pm	5	22	0.317	1.086	0.019	0.042	3.662	0.453	0.138	0.188	0.200	0.590	0.083	0.246	6.12	0.377	5.68
day 1 pm	5	23	0.414	1.261	0.013	0.040	5.965	0.331	0.184	0.139	0.238	0.911	0.119	0.343	7.11	0.537	9.70
day 1 pm	5	24	0.147	0.674	0.008	0.015	1.116	0.261	0.083	0.095	0.101	0.433	0.052	0.154	4.37	0.262	2.68
day 1 pm	5	25	0.138	0.986	0.012	0.020	2.620	0.455	0.117	0.091	0.243	0.480	0.080	0.212	6.71	0.308	6.79
day 1 pm	6	26	0.089	0.495	0.005	0.012	0.889	0.109	0.064	0.034	0.089	0.195	0.024	0.073	2.71	0.141	1.35
day 1 pm	6	27	0.116	0.623	0.003	0.017	0.881	0.115	0.070	0.030	0.088	0.395	0.017	0.042	2.94	0.226	1.03
day 1 pm	6	28	0.156	0.477	0.003	0.016	0.957	0.131	0.065	0.050	0.068	0.179	0.019	0.066	2.60	0.121	1.18
day 1 pm	6	29	0.163	0.754	0.006	0.014	0.887	0.207	0.082	0.048	0.146	0.319	0.042	0.117	3.88	0.201	1.47
day 1 pm	6	30	0.162	0.553	0.002	0.011	0.797	0.130	0.065	0.059	0.124	0.260	0.031	0.101	3.60	0.187	1.86
day 1 pm	7	31	0.165	0.347	0.012	0.013	0.420	0.055	0.037	0.063	0.098	0.161	0.015	0.100	1.69	0.120	1.13
day 1 pm	7	32	0.196	0.353	0.009	0.017	0.279	0.098	0.036	0.042	0.039	0.083	0.017	0.097	1.92	0.093	0.87
day 1 pm	7	33	0.068	0.173	0.005	0.008	0.143	0.044	0.016	0.024	0.043	0.064	0.007	0.057	0.86	0.047	0.49
day 1 pm	7	34	0.113	0.572	0.014	0.015	0.452	0.056	0.070	0.074	0.036	0.760	0.018	0.084	2.33	0.365	1.63
day 1 pm	7	35	0.212	0.580	0.013	0.023	0.276	0.103	0.070	0.106	0.068	0.337	0.015	0.071	2.61	0.231	0.83
day 2 am	1	1	0.110	1.389	0.016	0.026	2.414	0.157	0.004	0.074	0.319	0.220	0.057	0.185	8.03	0.063	4.30
day 2 am	1	2	0.085	0.905	0.004	0.015	2.242	0.170	0.003	0.038	0.112	0.299	0.044	0.182	4.52	0.078	4.48
day 2 am	1	3	0.066	0.676	0.007	0.014	1.688	0.214	0.003	0.044	0.142	0.267	0.040	0.217	3.27	0.090	3.44
day 2 am	1	4	0.068	0.799	0.006	0.016	1.771	0.173	0.030	0.049	0.168	0.750	0.037	0.103	4.73	0.140	4.30
day 2 am	1	5	0.102	1.066	0.013	0.021	2.386	0.159	0.006	0.038	0.164	0.302	0.045	0.234	4.84	0.077	5.76
day 2 am	2	6	0.109	0.892	0.010	0.018	4.528	0.167	0.005	0.065	0.128	0.335	0.068	0.446	4.54	0.164	8.15
day 2 am	2	7	0.055	0.756	0.005	0.013	1.719	0.142	0.005	0.031	0.141	0.272	0.037	0.075	4.21	0.070	3.48
day 2 am	2	8	0.072	0.836	0.004	0.017	2.627	0.106	0.004	0.025	0.162	0.198	0.029	0.059	4.37	0.044	4.67
day 2 am	2	9	0.056	0.580	0.005	0.011	2.268	0.101	0.004	0.021	0.071	0.125	0.032	0.095	3.74	0.052	4.22
day 2 am	2	10	0.086	0.856	0.013	0.021	1.948	0.134	0.003	0.086	0.148	0.165	0.043	0.166	4.36	0.043	5.13
day 2 am	3	11	0.086	1.176	0.011	0.018	0.168	0.000	0.000	0.071	0.000	0.148	0.024	0.178	5.15	0.130	2.85
day 2 am	3	12	0.109	2.632	0.019	0.023	0.076	0.000	0.000	0.058	0.000	0.150	0.036	1.238	7.91	0.488	3.66

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Sampling time	Group	Rat number	Glucose	Creatinine	Methylsuccinate	Alanine	2-Oxoglutarate	Succinate	Dimethylamine	N,N-Dimethylglycine	Betaine	Sucrose	Trigonelline	Xylose	Allantoin	3-Indoxylsulfate	Citrate
day 2 am	3	13	0.099	1.038	0.010	0.027	0.189	0.000	0.000	0.035	0.000	0.144	0.004	0.066	4.87	0.090	0.65
day 2 am	3	14	0.120	1.434	0.011	0.024	0.104	0.000	0.000	0.088	0.000	0.173	0.012	0.169	7.19	0.140	0.36
day 2 am	3	15	0.131	1.091	0.014	0.021	0.066	0.000	0.000	0.056	0.000	0.065	0.037	0.091	4.93	0.135	4.38
day 2 am	4	16	0.105	0.840	0.020	0.025	0.007	0.000	0.000	0.077	0.000	0.078	0.006	0.112	3.86	0.124	0.09
day 2 am	4	17	0.144	0.876	0.024	0.035	0.089	0.000	0.000	0.118	0.000	0.107	0.009	0.100	3.80	0.012	0.15
day 2 am	4	18	0.180	1.629	0.015	0.044	0.110	0.000	0.000	0.121	0.000	0.040	0.020	0.292	6.79	0.083	0.22
day 2 am	4	19	0.117	0.868	0.018	0.027	0.057	0.000	0.000	0.070	0.000	0.193	0.008	0.150	3.57	0.118	0.10
day 2 am	4	20	0.097	0.774	0.021	0.019	0.042	0.000	0.000	0.067	0.000	0.088	0.007	0.086	3.34	0.104	0.04
day 2 pm	1	1	0.218	1.226	0.026	0.043	5.262	0.425	0.005	0.246	0.328	0.406	0.106	0.378	8.54	0.068	6.86
day 2 pm	1	2	0.085	0.524	0.006	0.019	2.728	0.322	0.002	0.094	0.066	0.183	0.043	0.139	3.25	0.069	3.32
day 2 pm	1	3	0.148	0.956	0.011	0.026	3.975	0.590	0.004	0.185	0.256	0.428	0.105	0.363	6.35	0.103	5.63
day 2 pm	1	4	0.244	0.919	0.015	0.034	3.187	0.452	0.005	0.156	0.204	0.620	0.073	0.272	5.86	0.118	4.29
day 2 pm	1	5	0.175	0.976	0.019	0.036	3.454	0.400	0.004	0.103	0.171	0.348	0.086	0.218	6.23	0.137	6.45
day 2 pm	2	6	0.191	0.982	0.015	0.025	5.518	0.292	0.003	0.121	0.155	0.507	0.100	0.266	6.70	0.125	9.25
day 2 pm	2	7	0.101	0.510	0.008	0.014	1.525	0.269	0.002	0.056	0.079	0.306	0.034	0.082	3.05	0.067	2.52
day 2 pm	2	8	0.171	0.803	0.016	0.027	3.041	0.289	0.003	0.107	0.183	0.528	0.084	0.226	5.60	0.139	5.12
day 2 pm	2	9	0.101	0.647	0.009	0.015	3.450	0.236	0.003	0.058	0.106	0.188	0.048	0.141	4.63	0.071	4.68
day 2 pm	2	10	0.221	1.088	0.023	0.045	4.474	0.414	0.003	0.317	0.219	0.358	0.088	0.300	7.63	0.113	7.43
day 2 pm	3	11	0.225	1.534	0.020	0.026	0.066	0.000	0.000	0.080	0.000	0.210	0.014	0.147	5.18	0.129	0.22
day 2 pm	3	12	0.204	1.158	0.039	0.043	0.042	0.000	0.000	0.057	0.000	0.114	0.009	0.102	4.04	0.119	0.11
day 2 pm	3	13	0.231	2.159	0.017	0.045	0.117	0.000	0.000	0.042	0.000	0.276	0.008	0.129	6.80	0.133	0.26
day 2 pm	3	14	0.159	3.097	0.025	0.020	0.070	0.000	0.000	0.081	0.000	0.574	0.007	0.255	9.18	0.025	0.17
day 2 pm	3	15	0.039	0.369	0.010	0.010	0.000	0.000	0.000	0.025	0.000	0.085	0.008	0.030	2.21	0.053	0.22
day 2 pm	4	16	0.144	0.795	0.032	0.038	0.023	0.000	0.000	0.050	0.000	0.082	0.004	0.089	3.22	0.041	0.06
day 2 pm	4	17	0.574	1.590	0.079	0.094	0.031	0.000	0.000	0.121	0.000	0.236	0.011	0.216	5.17	0.114	0.15
day 2 pm	4	19	0.292	1.246	0.029	0.058	0.001	0.000	0.000	0.055	0.000	0.094	0.012	0.135	4.04	0.081	0.16
day 2 pm	4	20	0.204	1.158	0.039	0.043	0.042	0.000	0.000	0.057	0.000	0.114	0.009	0.102	4.04	0.119	0.11
day 3 am	1	1	0.132	0.925	0.010	0.025	2.926	0.202	0.126	0.060	0.175	0.073	0.057	0.449	4.83	0.112	5.44
day 3 am	1	2	0.053	0.672	0.004	0.015	1.689	0.084	0.077	0.021	0.082	0.021	0.022	0.065	3.15	0.024	2.01
day 3 am	1	3	0.060	0.347	0.004	0.010	1.334	0.147	0.049	0.032	0.069	0.016	0.022	0.110	1.71	0.060	2.07
day 3 am	1	4	0.092	1.411	0.012	0.020	2.596	0.197	0.195	0.047	0.231	0.041	0.037	0.073	7.20	0.101	3.90

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Sampling time	Group	Rat number	Glucose	Creatinine	Methylsuccinate	Alanine	2-Oxoglutarate	Succinate	Dimethylamine	N,N-Dimethylglycine	Betaine	Sucrose	Trigonelline	Xylose	Allantoin	3-Indoxylsulfate	Citrate
day 3 am	1	5	0.104	0.933	0.011	0.019	2.388	0.147	0.110	0.029	0.154	0.054	0.031	0.115	4.50	0.067	4.76
day 3 am	2	6	0.104	1.226	0.012	0.017	4.156	0.173	0.173	0.052	0.117	0.028	0.038	0.076	5.69	0.080	7.46
day 3 am	2	7	0.062	0.696	0.008	0.015	0.903	0.138	0.079	0.025	0.081	0.019	0.014	0.038	3.75	0.070	1.45
day 3 am	2	8	0.058	0.319	0.005	0.009	1.065	0.059	0.020	0.014	0.055	0.006	0.015	0.043	1.80	0.026	1.96
day 3 am	2	9	0.045	0.438	0.003	0.008	2.572	0.085	0.067	0.016	0.084	0.015	0.018	0.069	2.54	0.052	2.88
day 3 am	2	10	0.213	0.862	0.011	0.030	3.150	0.217	0.122	0.103	0.138	0.078	0.045	0.342	5.24	0.041	6.37
day 3 am	3	11	0.149	1.058	0.009	0.017	0.057	0.043	0.094	0.055	0.147	0.030	0.212	0.129	4.53	0.000	0.08
day 3 am	3	12	0.134	1.297	0.006	0.015	0.112	0.063	0.136	0.045	0.182	0.003	0.029	0.162	5.23	0.000	0.18
day 3 am	3	13	0.154	1.479	0.013	0.027	0.260	0.163	0.043	0.041	0.070	0.007	0.141	0.192	4.65	0.000	0.54
day 3 am	3	14	0.199	1.393	0.011	0.022	0.144	0.155	0.140	0.067	0.158	0.018	0.287	0.192	6.87	0.000	0.38
day 3 am	3	15	0.157	0.576	0.007	0.009	0.046	0.023	0.066	0.033	0.096	0.017	0.111	0.058	3.02	0.000	0.04
day 3 am	4	16	1.032	1.538	0.133	0.187	0.170	0.180	0.116	0.063	0.094	0.163	0.042	0.277	6.11	0.000	0.20
day 3 am	4	17	0.819	0.957	0.080	0.113	0.169	0.192	0.070	0.084	0.048	0.091	0.215	0.117	4.39	0.000	0.18
day 3 am	4	18	0.899	1.709	0.044	0.123	0.342	0.269	0.143	0.075	0.295	0.106	0.197	0.339	7.29	0.000	0.50
day 3 am	4	19	4.429	1.233	0.119	0.453	0.397	0.353	0.107	0.052	0.270	0.241	0.186	0.188	5.44	0.000	0.65
day 3 am	4	20	1.142	2.289	0.126	0.184	0.156	0.030	0.189	0.057	0.396	0.087	0.527	0.251	6.89	0.000	0.17
day 3 pm	1	1	0.296	1.390	0.026	0.032	6.565	0.425	0.162	0.245	0.310	0.101	0.170	0.431	9.94	0.107	9.98
day 3 pm	1	2	0.295	1.680	0.014	0.042	5.162	0.531	0.190	0.228	0.222	0.124	0.136	0.448	9.42	0.120	7.71
day 3 pm	1	3	0.170	0.757	0.012	0.026	3.578	0.810	0.153	0.180	0.223	0.053	0.071	0.269	5.17	0.098	4.50
day 3 pm	1	4	0.223	1.303	0.020	0.029	3.951	0.422	0.151	0.209	0.246	0.130	0.133	0.381	8.24	0.198	7.08
day 3 pm	1	5	0.221	1.190	0.022	0.028	2.898	0.336	0.128	0.115	0.222	0.097	0.104	0.234	6.96	0.264	6.40
day 3 pm	2	6	0.481	1.461	0.024	0.037	4.969	0.271	0.199	0.241	0.203	0.157	0.157	0.539	9.90	0.257	8.46
day 3 pm	2	7	0.275	1.356	0.018	0.035	2.718	0.471	0.151	0.209	0.308	0.093	0.135	0.405	9.33	0.326	4.30
day 3 pm	2	8	0.298	1.352	0.032	0.038	3.948	0.356	0.179	0.147	0.270	0.131	0.137	0.390	12.68	0.166	7.67
day 3 pm	2	9	0.257	1.089	0.016	0.024	5.712	0.265	0.144	0.106	0.222	0.087	0.112	0.255	7.50	0.196	7.52
day 3 pm	2	15	0.321	1.434	0.022	0.047	5.127	0.432	0.170	0.308	0.233	0.064	0.101	0.330	7.69	0.140	8.47
day 3 pm	4	16	3.386	2.020	0.198	0.597	0.272	0.142	0.159	0.086	0.332	0.360	0.133	0.308	7.41	0.000	0.27
day 3 pm	4	17	13.164	2.637	0.330	1.265	0.853	0.073	0.227	0.176	1.037	0.938	0.879	0.376	9.06	0.000	0.17
day 3 pm	4	18	2.224	2.206	0.063	0.318	0.484	0.269	0.184	0.112	0.369	0.294	0.613	0.409	7.62	0.000	0.46
day 3 pm	4	19	16.951	1.551	0.203	1.780	0.624	0.412	0.152	0.134	1.884	0.627	0.214	0.401	7.45	0.000	0.86
day 3 pm	4	20	4.137	2.867	0.175	0.463	0.400	0.060	0.264	0.070	0.825	0.193	0.095	0.163	9.97	0.000	0.18

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day 4 am	1	1	0.163	1.106	0.010	0.029	3.038	0.173	0.171	0.081	0.222	0.076	0.070	0.658	5.36	0.997	6.47
day 4 am	1	2	0.180	1.023	0.008	0.025	3.368	0.248	0.144	0.077	0.112	0.088	0.078	0.519	5.82	0.028	5.15
day 4 am	1	3	0.150	0.957	0.009	0.022	2.154	0.353	0.143	0.077	0.176	0.076	0.064	0.312	5.22	0.987	4.43
day 4 am	1	4	0.161	1.061	0.010	0.024	3.104	0.208	0.175	0.067	0.164	0.081	0.068	0.405	5.90	0.829	5.46
day 4 am	1	5	0.240	1.278	0.016	0.033	3.948	0.233	0.183	0.066	0.268	0.095	0.080	0.519	6.07	1.079	7.15
day 4 am	2	6	0.247	1.237	0.014	0.028	5.279	0.231	0.270	0.088	0.175	0.072	0.099	0.904	6.08	1.271	8.09
day 4 am	2	7	0.164	0.907	0.010	0.021	2.052	0.174	0.120	0.064	0.183	0.082	0.075	0.399	4.98	0.527	3.56
day 4 am	2	8	0.085	0.529	0.007	0.012	1.765	0.095	0.076	0.032	0.074	0.031	0.034	0.187	3.35	0.607	2.62
day 4 am	2	9	0.172	0.885	0.006	0.018	3.450	0.165	0.146	0.040	0.181	0.082	0.068	0.375	5.26	0.775	6.22
day 4 am	2	15	0.216	1.535	0.014	0.036	4.070	0.176	0.196	0.148	0.205	0.056	0.067	0.230	7.24	0.438	8.04
day 4 am	4	16	6.014	1.480	0.122	0.914	0.206	0.195	0.135	0.061	0.165	0.016	0.027	0.269	5.72	1.437	0.85
day 4 am	4	17	12.218	1.622	0.150	1.334	0.391	0.252	0.156	0.091	0.310	0.010	0.018	0.112	6.96	2.170	0.76
day 4 am	4	18	2.052	0.744	0.024	0.332	0.399	0.217	0.089	0.064	0.097	0.003	0.040	0.150	3.81	0.370	0.73
day 4 am	4	19	17.124	0.920	0.070	1.501	0.625	0.270	0.084	0.068	0.509	0.020	0.051	0.424	5.94	1.723	1.16
day 4 am	4	20	3.771	1.849	0.051	0.454	0.179	0.132	0.150	0.048	0.077	0.013	0.017	0.085	8.03	0.663	0.19
day 4 pm	1	1	0.397	1.028	0.021	0.040	6.597	0.393	0.150	0.207	0.199	0.078	0.125	0.387	7.50	1.085	7.47
day 4 pm	1	2	0.525	1.618	0.021	0.054	3.578	0.273	0.177	0.291	0.297	0.069	0.140	0.288	11.63	1.159	7.10
day 4 pm	1	3	0.226	1.205	0.009	0.025	3.247	0.271	0.115	0.116	0.176	0.083	0.114	0.254	6.96	0.037	4.49
day 4 pm	1	4	0.242	1.080	0.012	0.028	3.499	0.597	0.152	0.149	0.280	0.080	0.109	0.284	7.14	1.013	5.21
day 4 pm	1	5	0.265	0.993	0.008	0.028	3.258	0.185	0.073	0.111	0.168	0.075	0.104	0.237	5.89	0.874	6.29
day 4 pm	2	6	0.319	1.198	0.026	0.034	3.235	0.288	0.132	0.112	0.218	0.088	0.106	0.274	7.34	1.327	6.75
day 4 pm	2	7	0.424	1.505	0.023	0.033	5.942	0.303	0.186	0.182	0.170	0.153	0.173	0.548	9.92	1.783	10.51
day 4 pm	2	8	0.349	1.240	0.021	0.032	3.983	0.391	0.154	0.172	0.248	0.106	0.144	0.318	9.05	1.264	7.53
day 4 pm	2	9	0.327	1.182	0.028	0.029	3.354	0.230	0.108	0.127	0.267	0.055	0.119	0.324	7.78	1.359	6.91
day 4 pm	2	15	0.281	1.158	0.014	0.031	5.100	0.297	0.132	0.098	0.239	0.093	0.129	0.267	8.09	1.248	7.49
day 4 pm	4	16	13.9	0.948	0.070	1.200	0.951	0.332	0.078	0.084	0.555	0.010	0.066	0.261	6.58	1.69	2.07
day 4 pm	4	17	53.1	2.381	0.239	5.160	1.095	0.385	0.289	0.293	1.024	0.077	0.099	0.637	9.64	4.03	0.74
day 4 pm	4	18	8.72	1.776	0.053	1.039	2.816	0.496	0.187	0.253	0.561	0.038	0.123	0.434	11.39	1.04	3.43
day 4 pm	4	19	19.9	0.970	0.089	1.541	2.935	0.411	0.094	0.115	0.708	0.015	0.075	0.329	5.87	1.88	3.55
day 4 pm	4	20	15.0	1.901	0.097	1.339	0.841	0.250	0.177	0.138	0.412	0.063	0.101	0.442	13.40	1.37	0.97
day 5 am	1	1	0.168	1.142	0.015	0.030	5.456	0.295	0.219	0.108	0.253	0.087	0.092	0.713	5.11	0.69	9.33

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day 5 am	1	2	0.136	1.109	0.006	0.022	3.604	0.287	0.161	0.092	0.198	0.041	0.074	0.500	6.52	0.16	5.99
day 5 am	1	3	0.098	0.955	0.005	0.014	2.392	0.316	0.139	0.046	0.164	0.022	0.052	0.277	5.53	0.43	4.10
day 5 am	1	4	0.125	1.847	0.010	0.013	3.303	0.180	0.301	0.066	0.286	0.053	0.062	0.185	10.43	0.80	6.70
day 5 am	1	5	0.192	1.952	0.019	0.028	4.541	0.256	0.391	0.062	0.351	0.069	0.098	0.485	9.15	0.91	9.71
day 5 am	2	6	0.146	1.292	0.013	0.023	5.053	0.179	0.210	0.070	0.135	0.093	0.089	0.532	6.98	0.50	9.38
day 5 am	2	7	0.145	1.179	0.013	0.025	2.456	0.190	0.170	0.078	0.212	0.089	0.097	0.321	6.67	0.64	5.31
day 5 am	2	8	0.113	0.945	0.011	0.020	1.847	0.170	0.136	0.050	0.118	0.037	0.072	0.457	5.53	0.48	4.35
day 5 am	2	9	0.131	1.025	0.009	0.022	4.554	0.233	0.148	0.056	0.162	0.045	0.084	0.441	6.30	0.43	7.31
day 5 am	2	15	0.163	1.697	0.015	0.019	4.414	0.240	0.181	0.157	0.197	0.043	0.056	0.163	8.37	0.75	6.97
day 5 am	4	16	18.0	1.135	0.067	1.184	1.889	0.364	0.102	2.395	0.315	1.062	0.058	1.246	5.31	2.21	3.60
day 5 am	4	17	1.92	2.326	0.120	3.664	1.428	0.010	0.195	6.107	0.763	2.732	0.120	1.142	4.00	4.41	3.31
day 5 am	4	18	8.92	1.349	0.034	1.138	3.466	0.435	0.156	1.282	0.326	0.436	0.089	0.500	7.12	1.16	4.76
day 5 am	4	19	15.8	0.783	0.070	1.334	3.472	0.382	0.090	1.936	0.411	0.775	0.054	0.468	4.49	1.57	4.27
day 5 am	4	20	20.1	1.985	0.070	1.522	2.525	0.365	0.076	2.156	0.488	0.973	0.090	1.047	8.68	1.59	3.29
day 5 pm	1	1	0.286	1.254	0.027	0.033	6.635	0.508	0.161	0.247	0.234	0.082	0.159	0.465	8.77	0.58	9.75
day 5 pm	1	2	0.335	1.370	0.013	0.041	5.841	0.609	0.178	0.249	0.209	0.086	0.139	0.506	8.77	0.22	6.05
day 5 pm	1	3	0.220	1.098	0.017	0.021	3.885	0.598	0.147	0.163	0.237	0.049	0.113	0.314	7.04	0.38	5.42
day 5 pm	1	4	0.235	1.154	0.019	0.023	4.410	0.460	0.162	0.240	0.191	0.041	0.119	0.451	8.23	0.34	6.51
day 5 pm	1	5	0.255	1.045	0.025	0.026	3.687	0.454	0.121	0.149	0.200	0.050	0.101	0.312	6.45	0.47	6.21
day 5 pm	2	6	0.325	1.285	0.033	0.030	7.027	0.377	0.177	0.241	0.116	0.089	0.158	0.568	9.38	0.27	10.31
day 5 pm	2	7	0.198	0.782	0.019	0.020	3.687	0.371	0.116	0.177	0.167	0.012	0.094	0.257	6.10	0.29	5.58
day 5 pm	2	8	0.323	0.883	0.033	0.031	3.693	0.278	0.137	0.121	0.181	0.020	0.107	0.351	7.09	0.39	6.58
day 5 pm	2	9	0.205	1.040	0.020	0.021	5.891	0.399	0.145	0.142	0.205	0.050	0.109	0.337	7.51	0.42	8.11
day 5 pm	2	15	0.328	1.202	0.031	0.028	3.566	0.312	0.127	0.321	0.207	0.044	0.090	0.286	8.37	0.46	5.83
day 5 pm	4	16	19.6	0.943	0.089	1.505	5.171	0.409	0.092	2.301	0.581	1.017	0.077	0.396	5.16	2.20	4.91
day 5 pm	4	17	45.6	2.090	0.127	3.973	4.834	0.552	0.108	4.716	1.211	1.974	0.159	0.674	11.41	3.99	4.83
day 5 pm	4	18	13.5	0.964	0.066	1.750	3.648	0.439	0.113	1.784	0.557	0.700	0.097	0.310	6.82	1.45	4.77
day 5 pm	4	19	18.1	0.872	0.087	1.790	4.031	0.363	0.102	2.264	0.587	0.927	0.086	0.309	5.33	1.79	4.59
day 5 pm	4	20	32.3	1.941	0.143	2.626	6.989	0.538	0.045	3.025	0.890	1.729	0.150	0.498	11.20	3.08	8.13
day 6 am	1	1	0.168	1.142	0.015	0.030	5.456	0.295	0.219	0.108	0.253	0.087	0.092	0.713	5.11	0.69	9.33
day 6 am	1	2	0.136	1.109	0.006	0.022	3.604	0.287	0.161	0.092	0.198	0.041	0.074	0.500	6.52	0.16	5.99

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Sampling time	Group	Rat number	Glucose	Creatinine	Methylsuccinate	Alanine	2-Oxoglutarate	Succinate	Dimethylamine	N,N-Dimethylglycine	Betaine	Sucrose	Trigonelline	Xylose	Allantoin	3-Indoxylsulfate	Citrate
day 6 am	1	3	0.098	0.955	0.005	0.014	2.392	0.316	0.139	0.046	0.164	0.022	0.052	0.277	5.53	0.43	4.10
day 6 am	1	4	0.125	1.847	0.010	0.013	3.303	0.180	0.301	0.066	0.286	0.053	0.062	0.185	10.43	0.80	6.70
day 6 am	1	5	0.192	1.952	0.019	0.028	4.541	0.256	0.391	0.062	0.351	0.069	0.098	0.485	9.15	0.91	9.71
day 6 am	2	6	0.146	1.292	0.013	0.023	5.053	0.179	0.210	0.070	0.135	0.093	0.089	0.532	6.98	0.50	9.38
day 6 am	2	7	0.145	1.179	0.013	0.025	2.456	0.190	0.170	0.078	0.212	0.089	0.097	0.321	6.67	0.64	5.31
day 6 am	2	8	0.113	0.945	0.011	0.020	1.847	0.170	0.136	0.050	0.118	0.037	0.072	0.457	5.53	0.48	4.35
day 6 am	2	9	0.131	1.025	0.009	0.022	4.554	0.233	0.148	0.056	0.162	0.045	0.084	0.441	6.30	0.43	7.31
day 6 am	2	15	0.163	1.697	0.015	0.019	4.414	0.240	0.181	0.157	0.197	0.043	0.056	0.163	8.37	0.75	6.97
day 6 am	4	16	22.6	1.121	0.074	1.513	4.556	0.353	0.113	1.221	0.655	1.098	0.057	0.255	5.26	0.56	5.24
day 6 am	4	17	60.4	3.145	0.143	4.646	9.105	0.543	0.392	2.862	1.366	2.814	0.140	0.972	9.96	0.49	9.69
day 6 am	4	18	21.1	1.198	0.051	1.946	3.649	0.336	0.131	1.194	0.588	1.122	0.070	0.413	5.44	0.47	4.83
day 6 am	4	19	14.9	0.910	0.065	1.670	4.031	0.311	0.107	1.040	0.563	0.995	0.088	0.340	5.30	0.47	5.27
day 6 am	4	20	22.3	1.040	0.057	2.092	2.746	0.245	0.122	1.226	0.798	1.216	0.071	0.404	3.83	0.39	3.85
day 6 pm	1	1	0.286	1.254	0.027	0.033	6.635	0.508	0.161	0.247	0.234	0.082	0.159	0.465	8.77	0.58	9.75
day 6 pm	1	2	0.335	1.370	0.013	0.041	5.841	0.609	0.178	0.249	0.209	0.086	0.139	0.506	8.77	0.22	6.05
day 6 pm	1	3	0.220	1.098	0.017	0.021	3.885	0.598	0.147	0.163	0.237	0.049	0.113	0.314	7.04	0.38	5.42
day 6 pm	1	4	0.235	1.154	0.019	0.023	4.410	0.460	0.162	0.240	0.191	0.041	0.119	0.451	8.23	0.34	6.51
day 6 pm	1	5	0.255	1.045	0.025	0.026	3.687	0.454	0.121	0.149	0.200	0.050	0.101	0.312	6.45	0.47	6.21
day 9 pm	2	6	0.325	1.285	0.033	0.030	7.027	0.377	0.177	0.241	0.116	0.089	0.158	0.568	9.38	0.27	10.31
day 6 pm	2	7	0.198	0.782	0.019	0.020	3.687	0.371	0.116	0.177	0.167	0.012	0.094	0.257	6.10	0.29	5.58
day 6 pm	2	8	0.323	0.883	0.033	0.031	3.693	0.278	0.137	0.121	0.181	0.020	0.107	0.351	7.09	0.39	6.58
day 6 pm	2	9	0.205	1.040	0.020	0.021	5.891	0.399	0.145	0.142	0.205	0.050	0.109	0.337	7.51	0.42	8.11
day 6 pm	2	15	0.328	1.202	0.031	0.028	3.566	0.312	0.127	0.321	0.207	0.044	0.090	0.286	8.37	0.46	5.83
day 6 pm	4	16	19.1	0.855	0.078	1.376	4.030	0.346	0.111	1.036	0.793	0.852	0.087	0.218	5.35	0.28	5.03
day 6 pm	4	17	18.2	1.773	0.096	2.265	6.878	0.413	0.195	1.223	0.993	1.426	0.159	0.471	9.30	0.28	6.86
day 6 pm	4	18	15.3	0.728	0.060	1.562	2.389	0.209	0.089	0.832	0.688	0.744	0.083	0.218	4.92	0.35	4.19
day 6 pm	4	19	9.89	0.684	0.046	0.882	4.198	0.272	0.081	0.348	0.658	0.386	0.097	0.149	5.44	0.16	4.69
day 6 pm	4	20	25.5	1.065	0.085	2.054	3.635	0.244	0.133	1.296	0.740	1.262	0.122	0.264	6.41	0.44	5.49
day 7 am	1	1	0.101	0.930	0.010	0.022	2.581	0.140	0.000	0.050	0.178	1.279	0.055	0.519	4.28	0.08	4.79
day 7 am	1	2	0.144	1.359	0.010	0.022	0.674	0.118	0.000	0.035	0.164	2.009	0.075	0.318	7.27	0.22	1.82
day 7 am	1	3	0.099	1.113	0.009	0.019	2.278	0.226	0.000	0.045	0.216	0.568	0.056	0.243	7.16	0.10	4.24

Sampling time	Group	Rat number	Glucose	Creatinine	Methylsuccinate	Alanine	2-Oxoglutarate	Succinate	Dimethylamine	N,N-Dimethylglycine	Betaine	Sucrose	Trigonelline	Xylose	Allantoin	3-Indoxylsulfate	Citrate
day 7 am	1	4	0.079	0.561	0.008	0.016	2.553	0.205	0.000	0.058	0.093	0.417	0.044	0.256	2.69	0.08	3.51
day 7 am	1	5	0.111	0.882	0.013	0.026	2.174	0.227	0.000	0.032	0.213	1.618	0.045	0.427	3.83	0.12	3.40
day 7 am	2	6	0.165	1.359	0.016	0.021	3.373	0.141	0.000	0.075	0.153	2.534	0.096	0.641	5.58	0.15	7.24
day 7 am	2	7	0.147	1.142	0.006	0.007	0.979	0.104	0.000	0.052	0.190	1.335	0.084	0.251	5.64	0.10	2.63
day 7 am	2	8	0.122	0.870	0.013	0.025	1.429	0.218	0.000	0.029	0.135	1.081	0.067	0.398	4.04	0.15	3.04
day 7 am	2	9	0.118	0.746	0.012	0.022	5.318	0.298	0.000	0.045	0.135	1.340	0.059	0.395	3.34	0.13	5.42
day 7 am	2	15	0.084	0.675	0.011	0.015	1.728	0.127	0.000	0.075	0.108	0.546	0.030	0.174	3.17	0.07	3.11
day 7 am	4	16	12.6	1.417	0.069	1.112	8.821	0.283	0.192	1.009	0.342	0.686	0.076	0.439	6.96	0.02	7.34
day 7 am	4	17	5.78	1.826	0.033	0.822	8.845	0.263	0.246	0.572	0.289	0.371	0.136	0.836	7.48	0.15	7.07
day 7 am	4	18	7.74	0.692	0.031	0.853	2.952	0.165	0.108	0.933	0.296	0.361	0.077	0.234	3.76	0.10	3.96
day 7 am	4	19	2.91	0.775	0.024	0.369	6.691	0.306	0.111	0.292	0.200	0.125	0.099	0.242	4.84	0.08	5.40
day 7 am	4	20	11.6	1.064	0.041	1.239	5.784	0.274	0.153	0.981	0.393	0.584	0.058	0.170	4.63	0.06	5.46
day 7 pm	1	1	0.230	1.255	0.030	0.031	4.852	0.424	0.000	0.173	0.220	1.437	0.142	0.348	8.75	0.15	8.13
day 7 pm	1	2	0.238	1.315	0.013	0.034	3.848	0.356	0.000	0.167	0.141	1.406	0.132	0.288	9.09	0.17	6.92
day 7 pm	1	3	0.234	1.297	0.004	0.032	3.067	0.493	0.000	0.146	0.258	1.225	0.147	0.271	8.05	0.12	6.98
day 7 pm	1	4	0.187	0.567	0.013	0.022	3.170	0.331	0.000	0.120	0.110	0.591	0.064	0.203	3.60	0.05	3.83
day 7 pm	1	5	0.204	1.116	0.022	0.028	2.557	0.289	0.000	0.093	0.281	1.366	0.113	0.274	6.78	0.23	5.33
day 7 pm	2	6	0.289	1.248	0.037	0.036	4.312	0.472	0.000	0.213	0.173	2.232	0.137	0.546	7.90	0.11	9.06
day 7 pm	2	7	0.213	0.856	0.021	0.028	2.546	0.530	0.000	0.169	0.149	0.903	0.107	0.279	5.89	0.12	4.58
day 7 pm	2	8	0.228	0.687	0.021	0.026	1.674	0.270	0.000	0.076	0.094	0.480	0.069	0.295	4.38	0.06	3.49
day 7 pm	2	9	0.145	0.766	0.021	0.022	2.953	0.269	0.000	0.057	0.119	1.080	0.096	0.172	5.97	0.15	5.42
day 7 pm	2	15	0.373	1.395	0.036	0.052	3.620	0.475	0.000	0.334	0.246	1.406	0.110	0.332	8.41	0.21	5.87
day 7 pm	4	16	7.88	1.340	0.066	0.879	8.608	0.526	0.176	0.872	0.882	0.340	0.166	0.391	8.31	0.14	7.32
day 7 pm	4	17	1.89	1.594	0.039	0.327	10.033	0.588	0.213	0.555	0.596	0.168	0.214	0.594	8.94	0.17	7.57
day 7 pm	4	18	5.74	0.855	0.041	0.828	6.385	0.428	0.107	0.862	0.807	0.250	0.094	0.317	6.03	0.12	7.02
day 7 pm	4	19	1.03	0.743	0.026	0.152	6.280	0.538	0.123	0.363	0.616	0.044	0.132	0.292	5.86	0.09	6.60
day 7 pm	4	20	6.16	1.054	0.047	0.663	6.686	0.534	0.143	0.784	0.345	0.250	0.161	0.274	6.82	0.12	7.47