Pre-analytical (mis)handling of plasma investigated by ^{1}H-NMR metabolomics

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Abstract

The pre-analytical handling of plasma, how it is drawn, processed, and stored, influences its composition. Samples in e.g. biobanks often lack this information and consequently important information about their quality. Especially metabolite concentrations are affected by pre-analytical handling making conclusions from metabolomics studies particularly sensitive to misinterpretations. The perturbed metabolite profile, however, also offers an attractive choice for assessing the pre-analytical history from the measured data. Here we show that it is possible using Orthogonal Projections to Latent Structures Discriminative Analysis to divide plasma NMR data into a multivariate 'original sample space' suitable for further less biased metabolomics analysis and an orthogonal 'pre-analytical handling space' describing the changes occurring from pre-analytical mishandling. Apart from confirming established pre-analytical effects on glucose metabolization and the consequent increase in *e.g.* lactate and pyruvate, the sample preparation protocol involved methanol precipitation which allowed the observation of reversible changes in short-chain fatty acid concentrations as a function of temperature.

Keywords: sample handling, short-chain fatty acids, OPLS-DA

Introduction

Proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) offers some unique possibilities compared to the expanding universe of mass spectrometry metabolomics applications, namely the facile, directly quantitative and highly reproducible acquisition of metabolite data. Finding and characterizing metabolomic biomarkers important for human disease often require large sample cohorts, more so if an underlying confounding variability exists from both pre-analytical and post-analytical processing. In an ideal case, the variability in the data set should derive from original sample content rather than the sample handling since even in such a situation, a lot of observed variability is present naturally (such as biological or technical variability), unrelated to the research question at hand.

Knowledge about the quality of a stored biofluid sample in terms of how representative it is of the biofluid at the actual time of sampling is crucial but often overlooked. The surge in biobanking efforts and increase in analytical requests on biobanked material requires establishing proper preanalytical sample standard operating procedures assuring sample quality, not the least because a lot of the variability in clinical chemistry comes from errors in the handling process[1, 2]. The analytical method to be used in a given study is impacted differently depending on the preanalytical procedures that were used. Arguably, metabolomics is the technique most sensitive to differences in preanalytical handling as minute changes in metabolite levels can be detected and as such can be the consequence of slight sampling procedure variation. This was highlighted in recent work where samples from multiple biobanks were analyzed with NMR and the results showed that the differences between the biobanks (*i.e.* the preanalytical handling) were greater than the inter-individual variation[3]. Previous studies to assess quality of biobank samples have focused on different aspects of sample quality, such as preanalytical time to freezer[4-7], centrifugation speed[8] and/or temperature[4-7] as well as the effect of freeze-thaw cycles[7, 9] and storage at different freezer temperatures[10-12]. For recent reviews on effects of preanalytical handling on metabolite stability and recommendations, see Stevens *et al*.[13], Kirwan *et al.*[14] and Gonzalez-Dominguez *et al.* [15]. There is also an established preanalytical sample preparation standard, ISO 23118:2021.

NMR has previously been suggested as a good choice for quality assessment of biobank samples[16] and here, we report the effects of preanalytical temperature, time to centrifugation and light/no light on the ^{1}H NMR metabolome of plasma samples collected in K_2-EDTA tubes that were subjected to methanol precipitation before data acquisition. An LC-MS-based study of the same plasma raw material has been published[17].

Objectives

The present work aims to demonstrate the utility of using deproteinized plasma ^{1}H NMR spectra to describe the effects of pre-analytical handling such as varying the temperature, time to centrifugation and light/dark conditions on drawn blood samples by dividing the data into a multivariate 'original sample space' and an orthogonal 'pre-analytical handling space'.

Methods

Subjects

Blood samples were collected from 28 healthy volunteers, 19 females and 9 males, with ages ranging from 20 to 76 years old. All participants provided an informed consent prior to enrolling into the study, which was carried out in accordance with the Declaration of Helsinki and Swedish Biobank legislation. All volunteers were asked to participate twice, 13 did, giving in total 41 original samples. At the first occasion they were asked to give a 4-hour fasting sample prior to blood withdrawal and at the second visit they were asked to be in a non-fasting state. The study was approved by the regional ethics board in Gothenburg, reference number 054-15.

Plasma sampling and pre-analytical treatment

Unless otherwise stated chemicals were acquired from Merck. Blood samples were collected in K_2-EDTA 15.8 mg collection tubes including a gel separator (Vacutainer, Becton Dickinson). Every participant's blood ideally filled 24 tubes at each occasion, in principle giving 984 samples but for various reasons only 952 were collected for analyses. One of the samples was unusable meaning that the main study analyses comprised 951 samples. The tubes were stored at three different temperatures 4°C, 25°C or 37°C, during four different time periods of approximately 0h, 1h 30 min, 2h 30 min or 3h 30 min. Samples were kept either in darkness (tube covered in foil) or exposed to normal indoor room lighting before centrifugation at 2000g for 10 minutes. Samples stored at 4°C were centrifuged at 4°C and samples at 25°C or 37°C were centrifuged at RT. Samples were collected and pipetted into 96-well plates (REMP, Brooks Life Sciences) for cold storage (-80 °C). Additionally, eight EDTA tubes were taken at four separate sampling occasions from one participant. At each occasion, tubes were put at 4°C or 25°C for approximately 10min, 30min or 60min, or at 4°C for approximately 30min followed by 30 min at 25°C, or at 25°C for approximately 30min followed by 30 min at 4°C, followed by centrifugation at either 4°C or RT depending on what the last temperature incubation was.

Sample pre-processing

Plasma samples in a REMP plate were thawed at 4°C over night. 150 µl plasma per well was transferred to 96-well deepwell plates (DWP; Porvair cat. no. 219009, 2 ml polypropylene, square wells) using a Bravo 96-channel liquid handler (Agilent Technologies). Methanol precipitation was performed essentially as described in Gowda *et al.*[18]. Briefly, a Bravo liquid handler was used to mix 150 µl thawed serum with 750 µl cold (-20°C) methanol in the 96-well deepwell plate. The Bravo robot operated with 250 µl filter tips, necessitating several pipetting steps for the methanol addition. The plate was sealed with a piercable sealing cap (Porvair cat. no. 219004) and shaken at 12°C for 30 min at 800 rpm in a thermomixer (Eppendorf), placed at -20°C for 30 min and thereafter spun at maximum speed in an Eppendorf 5804R centrifuge with an A-2-DWP swing-out rotor (2250 x g) for 60 min at 4°C. 600 µl of the supernatant was transferred to a new deepwell plate with the Bravo liquid handler. After transfer was finished, the receiver plate was dried in a Labconco Centrivap (Labconco Corp.) lyophilizer at 20°C over night. The resulting pellets were first washed with 50 µl of deuterated methanol, shaken at 800 rpm, 12°C for 5 min with the thermomixer, dried in the lyophilizer again for 1h and then dissolved in 200 µl buffer A (37.5 mM sodium phosphate, pD 6.95, 100% D_2O, 0.02% NaN_3, 500 µM DSS-d_6, 1 mM imidazole) per well by shaking at 12°C, 45 min, 800 rpm in the thermomixer. Samples were spun down briefly before 180µL of each sample was transferred to 3 mm NMR tubes with a SamplePro L liquid handler (Bruker BioSpin). Samples were kept at 4-6°C during preprocessing and until data acquisition. For an overview of the sample preparation workflow, see Supplemental figure 1.

Additional K_2-EDTA plasma tube tests

A mixture of alanine, butyrate, propionate and acetate, each at 50 µM concentration in buffer A, with a total volume of 2 ml, was added to four EDTA tubes, inverted twice and incubated similarly as described above for the plasma samples, *i.e.* 1h at 4°C or 22°C, or 30 min each at 4° or 22°C in succession, both possibilities. A tube with only buffer A was also incubated at 22°C for 1h. After incubation, 600 µl of each incubated sample and a negative control of buffer A which had not been in contact with an EDTA tube was transferred to 5 mm SampleJet NMR tubes and sealed with POM balls (Bruker BioSpin).

NMR data acquisition

For methanol-precipitated plasma samples, 1D ^{1}H NMR data was acquired using an Oxford 800 MHz magnet equipped with a Bruker Avance III HD console and a 3 mm HCN cryoprobe. Sample racks were handled and kept at 6°C in a SampleJet sample changer before being put into the spectrometer. For 1D ^{1}H data, the pulse sequence 'zgespe' was used, entailing water suppression through excitation sculpting including a perfect echo element. The acquisition time was 2.04 s, the relaxation delay 3 s. Data was acquired in 128 scans and 64k data points. The spectral width was 20 ppm and the acquisition temperature 298 K. For the K_2-EDTA tube tests, a Bruker 600 MHz Avance III spectrometer equipped with a 5 mm BBI room temperature probe was used. 1D ^{1}H data was acquired with the 'noesygppr1d' pulse sequence using 32 scans collected into 64k data points, a spectral width of 20 ppm, an acquisition time of 2.726 s and a relaxation delay of 4 s for a total experimental time of 3 min 4 s. Acquired data was processed in TopSpin 3.5pl6 (Bruker BioSpin), including exponential line broadening of 0.3 Hz prior to Fourier transform, automatic phasing and baseline correction and referencing to the DSS-d_6 signal.

Analysis of spectral data

One participant completely lacked samples with short pre-analytical handling time and was therefore excluded, leaving 14 participants contributing with one sample and 13 participants contributing with samples from two occasions, giving in total 929 samples. Data was imported into MatLab 2020b (MathWorks Inc., Wilmington, USA), aligned with icoshift 1.2[19] and 325 peaks were visually localized and integrated down to an approximated baseline using in-house developed MatLab routines. The resulting integrated data was normalized with probabilistic quotient normalization [20] before import to SIMCA 17.0.0.24543 (Sartorius Stedim) for multivariate analysis. Principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) models were calculated within SIMCA and analyzed in SIMCA and MatLab. Integrated peaks were annotated with the use of ChenomX 8.3 (ChenomX Inc., Edmonton, Canada) and the human metabolome database (www.hmdb.ca)[21].

Results and discussion

Sample preparation

In contrast to our previous study using ^{1}H NMR on preanalytical effects on blood sampling[4], we chose to not employ the common standard operating procedure (SOP) of diluting plasma or serum samples but rather a methanol precipitation SOP adapted from Gowda et al. [18] to fit a workflow using deepwell plates to process large numbers of samples in parallel. The chosen SOP allows detection and quantitation of signals, *e.g.* short-chained fatty acids (SCFAs), otherwise masked by broad lipoprotein peaks when a dilution protocol is used.

EDTA tube contribution

The chosen K_2-EDTA tubes contribute propylene glycol, a molecule which is not present naturally in plasma which with this knowledge can be excluded from any study using the same tubes. However, the tubes also contribute formate, acetate and sarcosine, as well as signals from a number of unknown molecules (Supplemental figure 2). In accordance with previous studies[22, 23], this points to that EDTA plasma tubes,

especially those with a gel separator, are not ideal for ^{1}H NMR metabolomics. Serum or heparin plasma tubes are better choices for avoiding confounding signals.

Sample pre-analytical handling model

We realized from our data that *explicitly* setting plausible variables (*e.g.* time, temperature, light status) causing metabolite concentration changes can lead to biases, misinterpretations and inadvertently missing relevant factors. Therefore, we used OPLS-DA models with samples from each person and sampling occasion being its own group, rather than predefining preanalytical time and temperature in the model building. We decided that all samples with pre-analytical sampling times less than 15 minutes, which in practice in almost all cases meant less than 5 minutes, could be considered good and representative of the original sample space. For each participant, the mean intensity of all variables of these samples were calculated and used as mirror points (see Supplementary material). In this way each participant got one 'mirrored sample' per original sample and the model comprised in total 929+929 samples. The score and loading space then represent the original sample space, while the orthogonal score and loading space represent changes due to pre-analytical handling. We think a more traditional linear model rather than a non-linear (*e.g* AI) is reasonable since the work presented, and likely also future work, must restrict itself in number of samples and therefore also in precision. In addition, OPLS-DA has the advantage of an explicit, easy to interpret output in terms of scores and loadings. Describing the changes related to pre-analytical handling of a sample as following a trajectory in a multidimensional space of metabolite concentration orthogonal to a space of original sample concentration combinations is attractive due to its simplicity, making OPLS-DA a good choice. The models were built on univariately scaled and centered data since we think it makes sense that errors relate to between-sample variability rather than absolute numbers.

An extensively overfitted model using 39 components to maximize the size of a multivariate room spanning the 40 original samples was built to describe the original sample space (Figure 1 and Supplemental figure 6). The large number of components maximizes R2 minimizing any remaining information outside the model and maximizes the possible number of independent variables reducing the risk of non-true metabolite concentration correlations between these. Q2 is not describing anything relevant, both the accuracy and precision can best be estimated from predictions when using the model. Already within the model it is possible to get an approximative idea about its precision though, independent of their pre-analytical handling each person's samples should be positioned almost at the same place in score space, pushing pre-analytical handling related changes into the orthogonal space similar to all persons. Also, back calculated metabolite concentrations should be consistent for each person's samples. Indeed, adding orthogonal scores show consistent pre-analytical handling patterns between persons. The first two orthogonal components capture most of the temperature and time effects while adding a third captures methanol precipitation dilution variability and data normalization. Adding more components had limited impact. The fourth does not seem to have an obvious preanalytical explanation. The fifth and sixth orthogonal components show a pre-analytical handling effect at especially 37°C unique for some, especially one, person due to a large increase in one unknown metabolite with peaks at 1.36/28.75 ^{1}H/^{13}C ppm and 3.30 ^{1}H ppm. The ambient light conditions during sampling did not have any obvious effect on the model. Each persons samples gather together in the scores of the model, the ones being more in the center seemingly randomly depending on pre-analytical handling while some original samples with more deviating metabolite patterns still have small but obvious correlations with pre-analytical handling (not shown).

Back-calculating the individual molecule concentrations either from the model or from subtracting the original data with the orthogonal model shows that six orthogonal components largely suppress pre-analytical handling effects in the data (Figure 6a,c and Supplemental figures 7-20a,c). Prior modelling most peaks show small pre-analytical handling differences in intensity although it could be expected that many metabolite concentrations don't vary at all during the up to four hour pre-analytical handling period. Probably this can be attributed to the precision in the intensity calculations in combination with the normalization of the data after methanol precipitation with most normalization constants between 0.9 and 1.1 but some deviating up to 30% from average making otherwise quantitative NMR measurements less so. This spills over into the model with many metabolites having small deviations from zero in the orthogonal loadings risking minor accuracy problems of the corresponding back calculated metabolite concentrations. A more robust protocol, *e.g.* as would be possible from using the Bruker IVDr SOP, involving a stricter quality control, would probably be beneficial also for concentration estimations after modeling. In fact, even three orthogonal components are sufficient to obtain relatively good precision in the pre-analytical handling effect estimations (not shown). Also in a larger study a smaller number of orthogonal components should be sufficient to describe the major pre-analytical handling effects as long as it is not enlarged by other surprising effects like the 1.36/3.30 ppm molecule mentioned above or samples with very different pre-analytical handling metabolite concentration change patterns *e.g.* due to disease.

The main model, as well as separate models of each temperature, show four distinct temperature-dependent pre-analytical handling characteristics for some SCFAs, the glycolytic intermediate pyruvate, glucose, and a group of molecules including lactate and ornithine (Figure 2). The glucose, lactate, pyruvate and ornithine changes are consistent with earlier studies[4, 6, 24]. At 4°C essentially nothing happens except for an initial quite dramatic, decrease in SCFAs and pyruvate (Figure 2a,d). The orthogonal scores of the 5 min samples are separated from the others. At 25°C the SCFAs also decrease but to lesser extent (Figure 2b,e). Pyruvate, lactate and ornithine increase and glucose decreases. The 5 min, 1.5 h and 3.5 h samples are separated in orthogonal space with the 2.5 h overlapping both the 1.5 h and 3.5 h samples giving an idea about how fast the metabolite concentration changes are and the resolution of the model, *i.e.* how precise it is possible to determine the pre-analytical time before centrifugation. At 37°C glucose decreases the most, SCFAs only slightly (Figure 2c,f) and pyruvate, lactate and ornithine increase more rapidly than at 25°C. The 2.5 h samples are at this temperature separated from both the 1.5 h and 3.5 h samples.

Prediction modeling

We predicted all samples' metabolite concentration changes and applied adequate corrections, for each of the 14 participants who had samples only from one occasion, using separate OPLS-DA models with 38 regular and 6 orthogonal components where the predicted participants' data were excluded. The orthogonal components successfully subtract the differences related to pre-analytical handling between each person's samples. The 'pre-analytical handling direction', *i.e.* 'the derivative' of the orthogonal space, is almost intact and each persons predicted sample positions closely localized in the model plane. The precision of metabolite concentration predictions is also good. But leaving a person's samples outside a model predicting these instead result in consistent biases in both the components and the orthogonal components (Figure 3 and Supplemental figures 3, 4).

The prediction errors also translate into errors in the predicted metabolite concentrations (Figure 6b,d and Supplemental figures 7-20b,d) motivating a more careful examination into causality to find possibilities for improvement. The 325 variables used is larger than the underlying number of measured metabolites and therefore not determining the dimensions of the system. But the metabolites are clearly more than the 39 groups spanning the OPLS-DA models, meaning that the OPLS-DA models assume linear dependencies between the metabolites such that new predicted samples can be outside the model plane with a remainder not correctly predicted. Preferably the number of model dimensions should reflect the number of metabolites which is approximately twice compared to now. But most importantly, the model would gain from additional samples, being less sparse and with a wider distribution. There is now an obvious correlation between the distance to model and Hotelling's T2, versus the concentration accuracy in predictions (Supplemental figure 5). Good predictions are obtained when there are samples that are relatively similar in the model compared to the predicted ones. Since the idea is to replace a set of measured concentrations of an individual, which could be thought of as a position in a multivariate space, with another coordinate in this space but within the original sample region, both the pre-analytical handling trajectory to follow into the allowed space and the shape of the space itself needs to be relatively correct. The trajectory, the orthogonal components, seem to be well predicted using data from only a few individuals and future models mostly need data without pre-analytical handling bias to define the score space of the allowed region with higher precision. Preferably this data should be drawn from a wide distribution of people also including unusual sample types such as less controlled diabetics.

The risk of metabolite concentration accuracy errors should be weighed against the possible gains from prediction. Already our rather crude model, with relatively few underlying samples at hand, shows that glucose, lactate, fucose, ornithine and taurine gain from prediction, when predicted time to centrifugation is 2.5 hours or more at 37°C but also at 25°C. This holds as long as the distance to model and Hotelling's T2 does not differ a lot compared to the samples in the model. Other molecules such as 2-oxoisocaproate, acetate, choline, glutamine, mannose, propylene glycol and pyruvate would also be beneficial to predict using a model with more samples as outlined above. Already from the present model, however, qualitative estimates of these measured concentrations can be given as 'probably too high' or 'too low'.

Analysis of temperature and time effects on SCFAs

The decrease of some SCFAs, especially propionate and butyrate, prompted additional investigation (Figure 4). The decrease was most pronounced at 4°C. The extra experiments with alternating temperature showed that the decrease observed in SCFAs at 4°C is reversible, *i.e.* metabolite intensity is partly recovered by increasing the temperature to 22°C prior to centrifugation. The decrease is rapid since the differences are seen already at the first measurement point after ~10 min. The decrease in SCFAs at cold temperature is not seen in the control experiment with a mix of SCFAs alone without plasma in the same type of EDTA-plasma collection tube (Supplemental figure 2a). The reversibility of the changes seen in SCFAs suggest that it is a real biological effect connected to interaction with plasma macromolecules, *e.g.* proteins and/or cellular membranes. If this is a general phenomenon as a response to cold temperature, it might be an indication that certain SCFAs are used as natural cryoprotectants, a finding that needs further investigation and is outside the scope of this work. The concentration variability of SCFAs depending on the temperature in a sample is from the analysis perspective worrying. It is obvious that these concentrations are often underestimated since the temperature just prior to

sample processing often is lower than body temperature if tubes have been stored in a fridge or even in room temperature. If this temperature is not the same for all samples in a study it could easily introduce a bias.

Analysis of light exposure effects

It is well known that bilirubin in plasma can be affected by light exposure but only after extended storage at ambient or above ambient temperature[25]. To the best of our knowledge, no report has been published on the effect on plasma metabolites due storage in ambient light. It is true that light exposure in the present work does not seem to have any effect in the pre-analytical handling estimation and prediction models,
but has a minor effect when specifically comparing matched light-dark vs dark-light sample pairs in OPLS-DA models. 2-oxoisocaproate and 3-hydroxybutyrate are relatively higher and lower in concentration, respectively, when exposed to light (Figure 5). Permutation tests, and models on different subsets leaving the remainder as independent data to test on, show that these differences are real. The result is consistent with various model subgroups of people, temperatures, and times to centrifugation (not shown).

Other considerations

To avoid misinterpreting any measurement batch effects for preanalytical sample handling changes, all samples, with few exceptions, were randomized for each person and occasion and measured immediately after each other. Consequently, the dataset does not allow any precise batch effect control and instead batch variation will go mainly into the individuals' modelled scores. We do not think there is any reason to believe that we have any major batch effects, but it should be noted that we for a very small number of peaks saw a change in peak intensity depending on time in the cooled SampleJet refrigerator prior to NMR measurement.

Limitations of the present study

A bigger dataset comprising more than the 27 unique individuals in this work is needed to make models suitable for assessment of existing plasma samples in biobanks, covering not only a bigger 'person-space' but also disease states, different sampling tubes *etc*. However, as long as the model incorporates representative samples, the approach taken here is valid to use as an assessment of sample status at the time of freezing.

Conclusions

The pre-analytical handling metabolite concentration change patterns, ‘the derivative’ of sample concentrations, are largely conserved between samples. Biologically allowed ratios between certain metabolites when a sample is drawn, the relative metabolite concentrations at ‘time zero’, are also limited. This makes prediction of at what temperature and for how long a plasma sample has been handled before centrifugation possible. We show that it is possible to predict metabolite concentrations of a corresponding original sample using an OPLS-DA model, making comparisons between differently handled samples possible and analyses of biobanked samples from different sources more feasible than today. In this context it is tempting to suggest future models used by the community, built on a large shared dataset where relevant subsets can be used when motivated, for example depending on what sampling tube type was used.

If samples are stored in ambient light or not prior to centrifugation has no practical significance for all metabolites with the exceptions 2-oxoisocaproate and 3-hydroxybutyrate. However, if SCFAs are of interest in a

study, the recommended sampling SOP should be to keep sample tubes at room temperature before centrifugation to ensure correct SCFAs concentrations without unnecessary pre-analytical handling. Without knowledge about the actual sampling protocol used for a given sample set requested from a biobank, any resulting quantification of SCFAs (including acetate in the case of EDTA-plasma) should be handled with caution as there might be confounding bias due to sampling conditions. In an ideal case but still practical in terms of everyday work in clinical chemistry labs, plasma sampling should be done immediately at RT, otherwise storage at 4°C followed by warming to RT just before centrifugation is desirable to represent the metabolome reasonably accurately to the state it was at blood draw. The warming of sample tubes before centrifugation goes against current recommendations[14, 15, 26] but it also the first time to our knowledge that the decrease of SCFAs in plasma at low temperature has been observed.

With the present work and previous studies, the inherent reproducibility and quantitative aspect of ^{1}H NMR spectroscopy has demonstrably shown its suitability for facile quality control of serum and plasma pre-analytical variability. However, there is no established standard on sample preparation SOP or comprehensive models taking into account all potential variables at play, *i.e.* matrix, sampling tube, temperature, centrifugal force *etc*. What we propose in this work is that all pre-analytical variability affecting the metabolome can be captured in the orthogonal space without explicitly stating what a given sample has been subjected to. Thus, a future sample can be classified in this 'pre-analytical handling space' as one which displays a metabolite profile consistent with *e.g.* 25°C storage for 2h or 37°C for a shorter time, even though the actual conditions were not known. To be adopted into practical use, however, this approach requires the model to be extensively expanded to cover other variables such as the mentioned tube types, serum as well as different types of plasma, and representation of not only healthy subjects but also disease states.

Conflict of interests

The authors declare no conflicts of interest.

Author contributions

ÅTN and LP conceived and designed the project. HZ performed the pre-analytical handling experiment. ABN and DM performed the sample preparation and data acquisition of NMR data for both human samples and tube tests. ABN, DM and GK analyzed the NMR data. ABN, DM, GK and ÅTN wrote the manuscript. All authors read and approved the manuscript.

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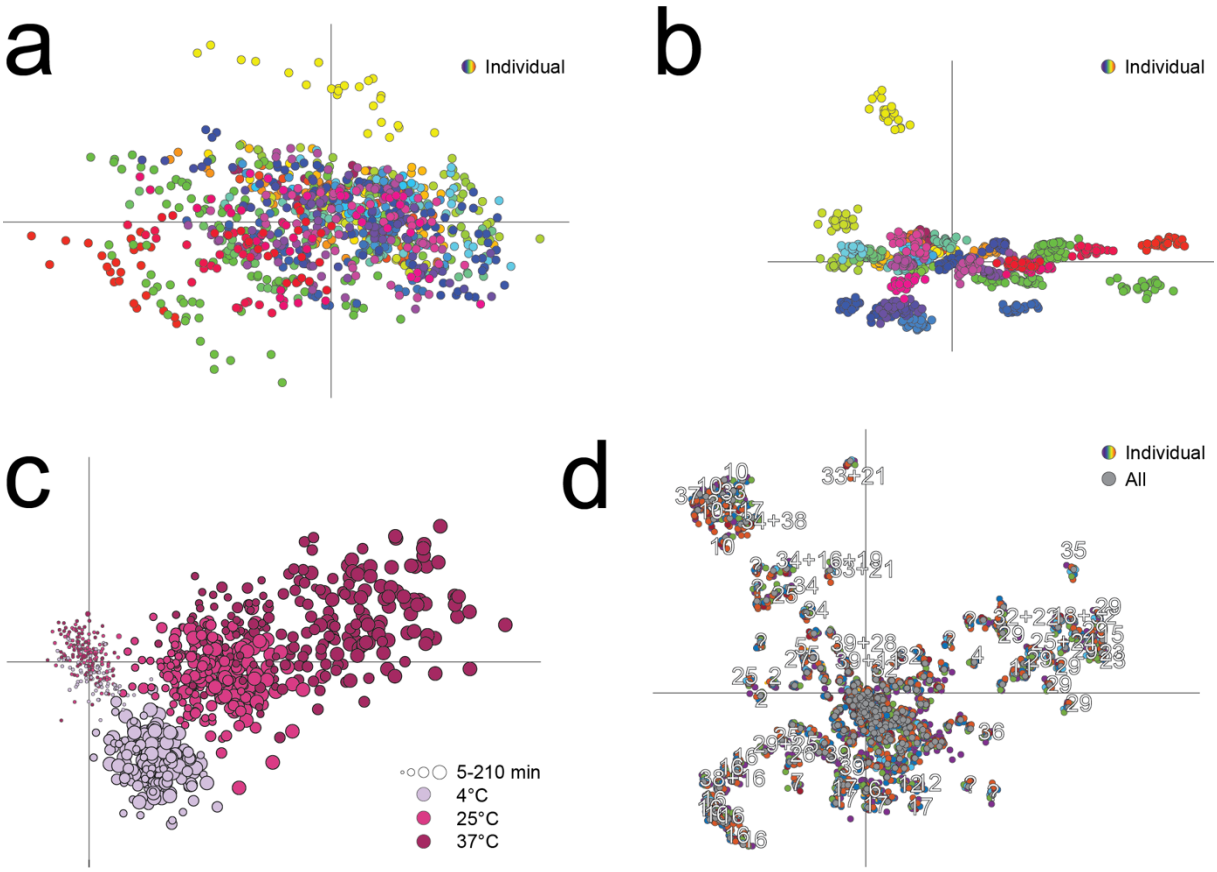

Figure 1: *A 39 component, 6 orthogonal component OPLS-DA model, discriminating according to the 40 sampling occasions of 27 participants, of 325 peak intensities from 929 plasma spectra exposed to different pre-analytical incubation times at different temperatures either in the dark or in ambient light.* ***a)*** *The first two component scores in a PCA, without the 'mirrored data', illustrating the uncorrected changes occurring during incubation. Samples from a given individual are relatively well localized but the pre-analytical incubation time and temperature trends induce scattering.* ***b)*** *The first two component scores of the corresponding OPLS-DA model when also the 'mirrored sample' data is used. For simplicity, the mirrored samples are excluded from the plot. The score space is a lower dimensional projection of the original data matrix and as such to a large degree filtered from inconsistencies in the induced changes. The positions of the samples are close to where they would have been if not exposed to the induced changes from incubation.* ***c)*** *The first two orthogonal component scores, with 'mirrored samples' excluded from the plot. The mirrored sample strategy assure that samples with short incubation times display low orthogonal score values while longer incubation times give larger orthogonal scores. The patterns of changes are similar for all individuals.* ***d)*** *The corresponding loadings (grey dots) show the metabolite patterns for induced changes. 14 separate models for prediction were made for the 14 participants who only contributed samples from one occasion by excluding one individual at a time. The first two orthogonal components of these models remained similar (colored dots). Numbering refers to identified metabolites listed in Supplemental table 1.*

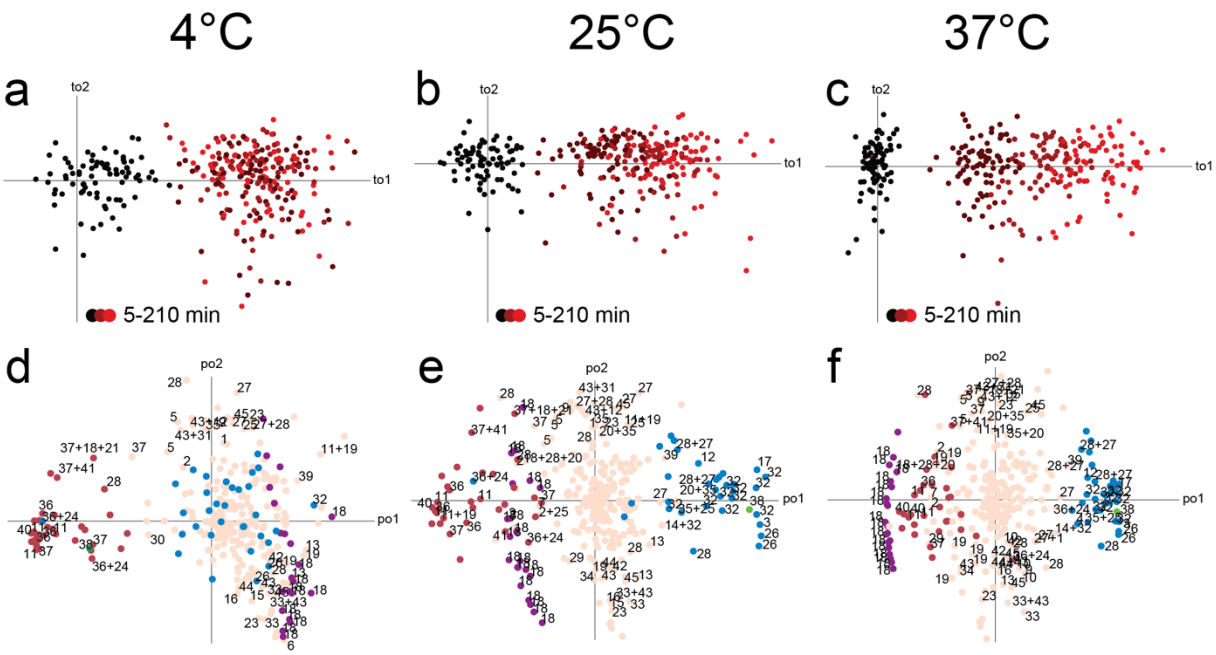

Figure 2. *Scores (**a–c**) and loadings (**d–e**) of OPLS-DA models of 4°C, 25°C and 37°C data show four distinct groups of characteristic changes depending on incubation temperature; short chain fatty acids (red), the glycolytic intermediate pyruvate (green), glucose (purple) and a group of molecules including lactate and ornithine (blue). Numbering is according to Supplemental table 1.*

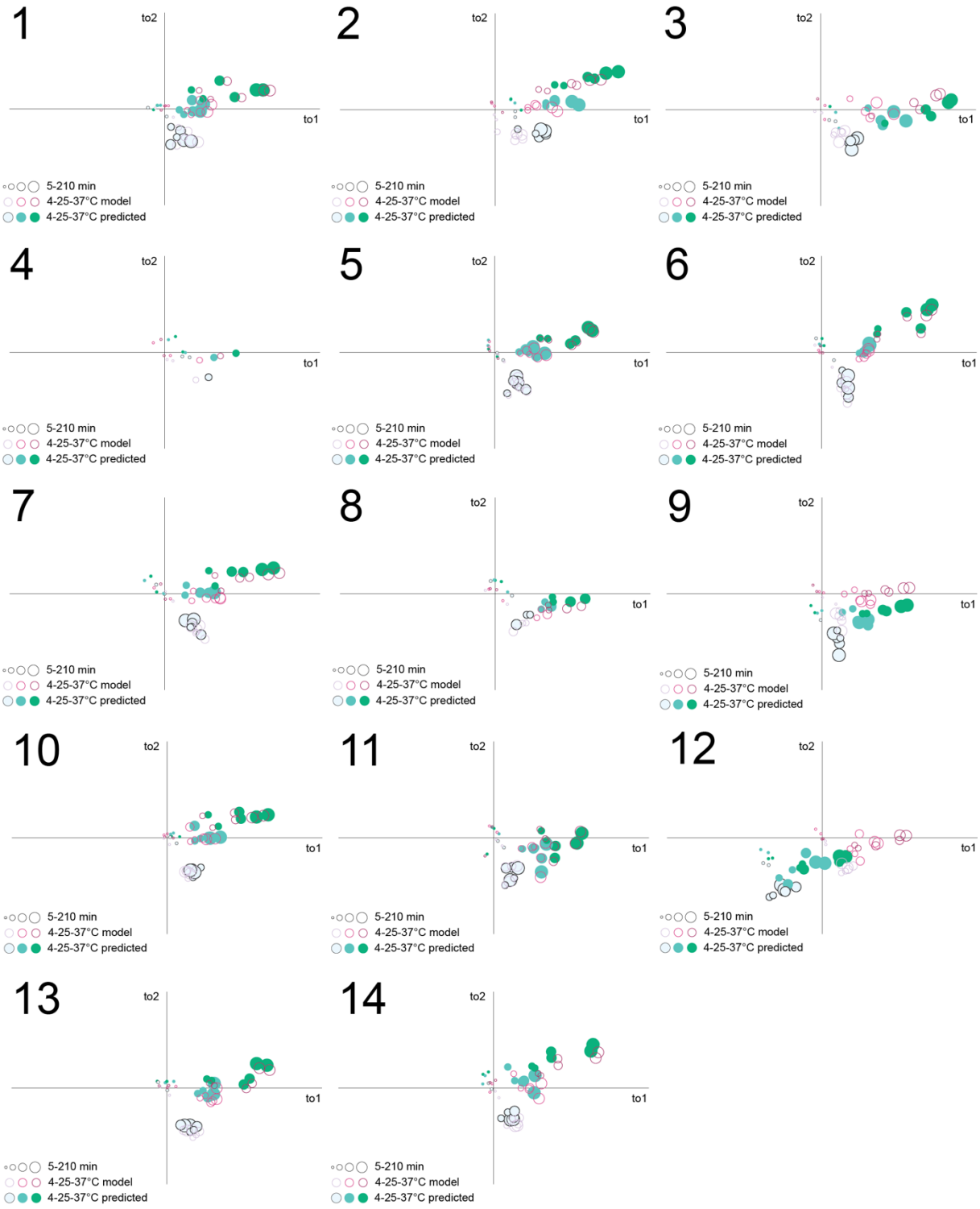

Figure 3. *The first two orthogonal scores of separate prediction models for the 14 participants who contributed with samples from one occasion only. The relative positions between the orthogonal scores for each participant whether the samples are included in the OPLS-DA model or excluded and predicted are almost conserved, meaning that the orthogonal score 'pre-analytical handling space' is well described. When the predicted data of an individual does not fit well into the model in terms of distance to model and Hotelling's T2 range,* e.g. *participant 12, also the orthogonal scores tend to be biased.*

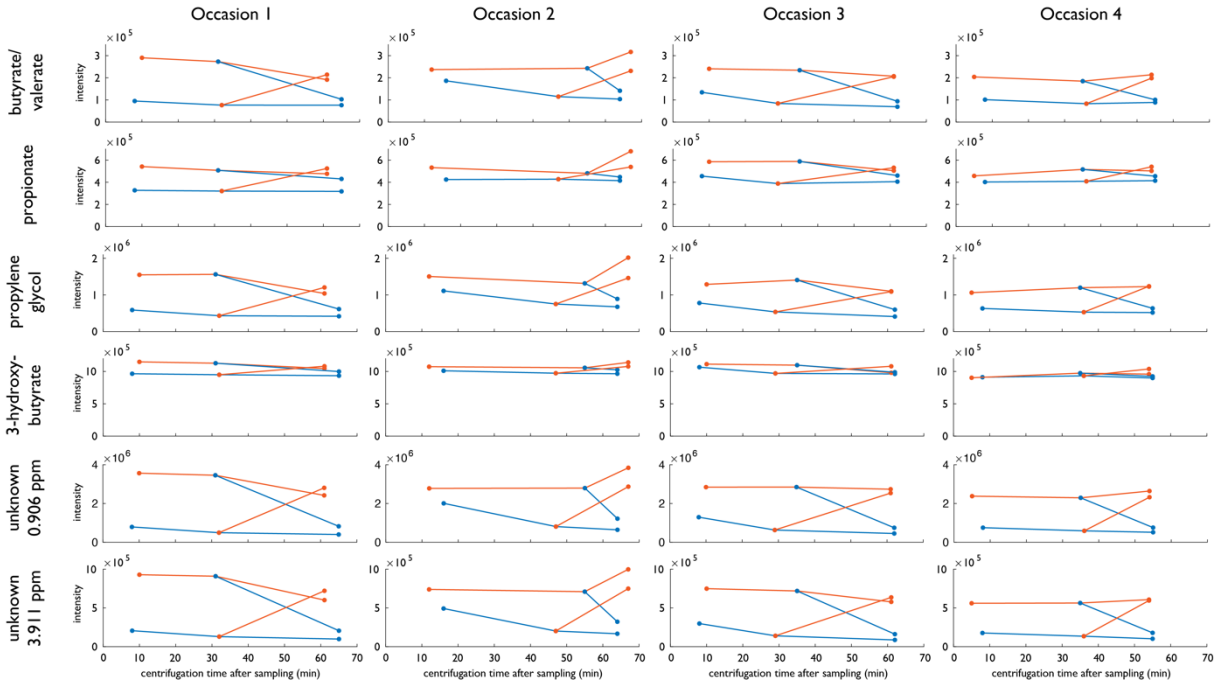

Figure 4. *Effect of incubation at cold temperature before centrifugation. Samples were placed at 4°C (blue) or 22°C (red) for approximately 30min, 60min or a sequence of 30+30 min at 4°C followed by 22°C, or vice versa. Butyrate/valerate, propionate, propylene glycol, 3-hydroxybutyrate and an unknown molecule decrease at 4°C but at least partly recovers at 22°C.*

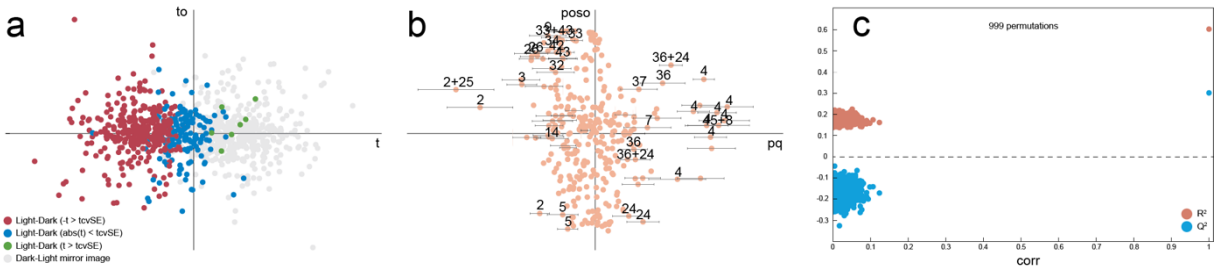

Figure 5. a) *The score plot of an OPLS-DA model of Light-Dark (red, blue or green, depending on size compared to the cross validated standard error) vs. Dark-Light (grey) of all 470 available light and dark sample pairs show a minor separation of the groups.* ***b)*** *The corresponding loading plot show 2-oxoisocaproate (2) increase and 3-hydroxybutyrate (4) decrease in ambient light compared to the dark. The error bars show the cross validated standard error.* ***c)*** *Cross validation was performed using seven groups, always including samples from the same person in the same group. A permutation test shows a much higher cumulative Q^2 value for the model than in any of 999 permutations.*

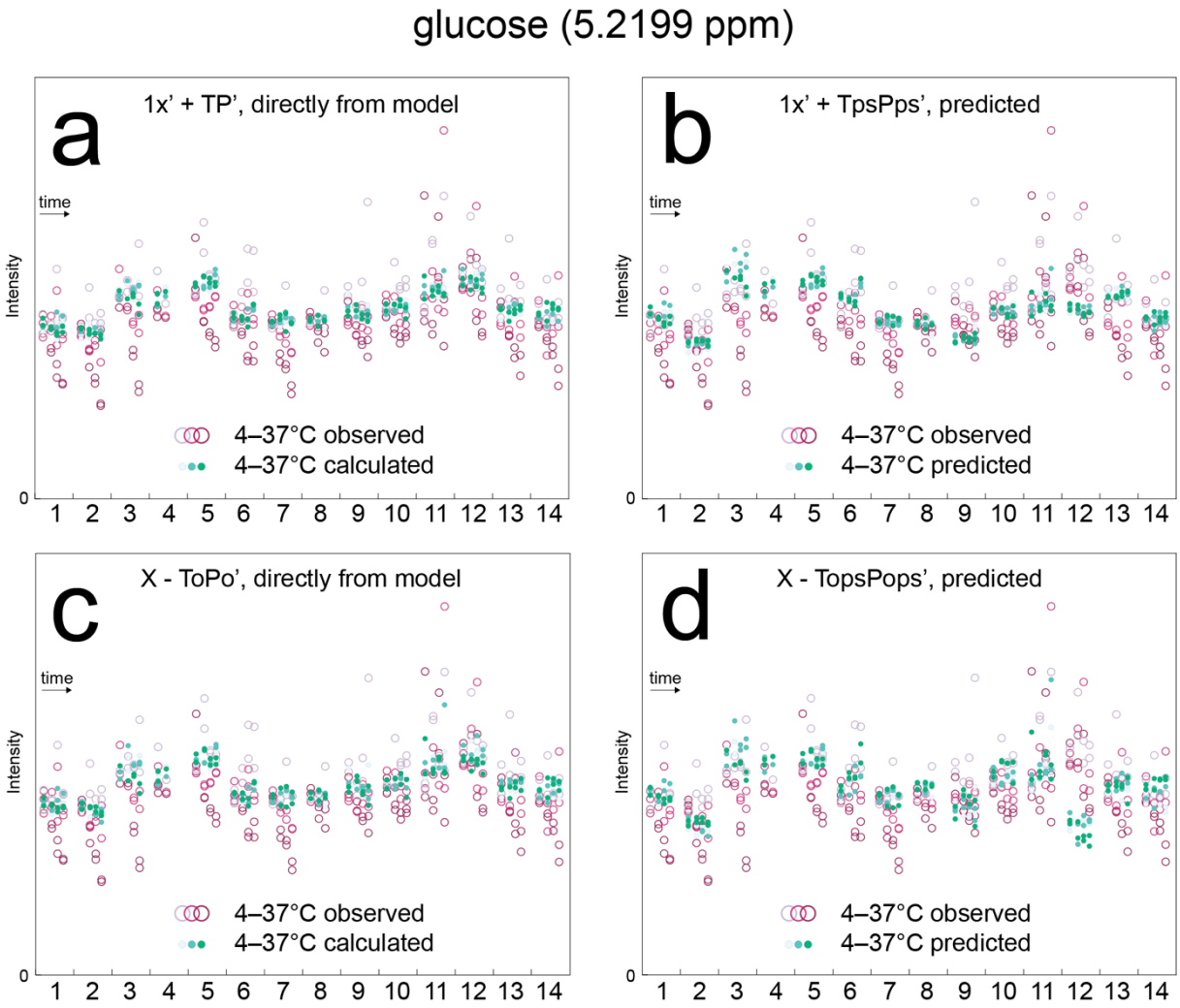

Figure 6. *The original metabolite concentrations of the 14 participants with samples only from one occasion were calculated directly from* ***a)*** *the components and* ***c)*** *orthogonal components of the original model, as well as predicted from* ***b)*** *the components and* ***d)*** *orthogonal components of the corresponding model where the participant was excluded. Here, glucose is shown (see Supplemental figures 7-20 for other metabolites). The calculated values are consistent for each participant and also agrees well with the observed values for non-incubated samples, independent of using scores or orthogonal scores. The predicted values are also consistent for each participant but often contain a bias for those participants with large distances to model and Hotelling's T2,* e.g. *participant 12, making predictions only suitable for those close to the model plane.*