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Long-term stability of human plasma metabolites during storage at -80 °C

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KEYWORDS

biobanking, metabolomics, long-term stability, human plasma, storage, mass spectrometry

ABSTRACT

Prolonged storage of biospecimen can lead to artificially altered metabolite concentrations and thus bias data analysis in metabolomics experiments. To elucidate the potential impact of long-term storage on the metabolite profile, a pooled human plasma sample was aliquoted and stored at -80 °C. During a–time period of five years, 1012 of the aliquots were measured with the Biocrates Absolute*IDQ*® p180 targeted-metabolomics assay at 193 time points.

Modelling the concentration courses over time revealed that 55 out of 111 metabolites remained stable. The statistically significantly changed metabolites showed on average an increase or decrease of +13.7% and -14.5%, respectively. In detail, increased concentration levels were observed for amino acids (mean: +15.4%), the sum of hexoses (+7.9%), butyrylcarnitine (+9.4%), and some phospholipids mostly with chain lengths exceeding 40 carbon atoms (mean: +18.0%). Lipids tended to exhibit decreased concentration levels with the following mean concentration changes: acylcarnitines: -12.1%, lysophosphatidylcholines: -15.1%, diacyl-phosphatidylcholines: -17.0%, acyl-alkyl-phosphatidylcholines: -13.3%, sphingomyelins: -14.8%.

We conclude that storage of plasma samples at -80 °C for up to five years can lead to altered concentration levels of amino acids, acylcarnitines, glycerophospholipids, sphingomyelins and the sum of hexoses. These alterations have to be considered when analyzing metabolomics data from long-term epidemiological studies.

INTRODUCTION

Metabolomics analyses of clinical cohort studies revealed new biomarkers and gave new insights into aetiology and biochemical mechanisms of epidemic diseases like type 2 diabetes [1–3], cardiovascular diseases [4–6], and cancer [7,8].

However, for successful study outcomes a number of technical challenges and pitfalls have to be addressed at all steps of study design. A major challenge is to minimise the potential introduction of bias during the pre-analytical, analytical, and post-analytical phase. Notably, a considerable amount of the artificially introduced errors occur during the pre-analytical study phase[9] and improvident sample handling can have detrimental effects on sample quality [10–15] Ellervik and Vaught identified sample collection, processing, transport, and storage as the most relevant technical factors during the pre-analytical phase [16].. Several markers (DNA, RNA, proteins, metabolites) allowing quantification of sample deterioration in different matrices have been reviewed recently in Lee *et al.* [17]. Some metabolites seem to be rather stable; others react very sensitive to perturbations. Ascorbic acid (Vitamin C) as an example for a stable metabolite was found to be not significantly changed in its concentration over 11 years of storage in liquid nitrogen [18]. As part of the parameter LacaScore, which comprises the ascorbic acid to lactic acid ratio, ascorbic acid thus can be used to quantify the quality status of plasma samples [19]. On the other hand, metabolite classes like poly unsaturated fatty acids, oxylipins [11,20], and glycerophosphatidylcholines [12,14] react very sensitive to improper sample handling.

While sample collection, processing, and transport can principally be controlled and standardised, alterations of metabolite concentrations during storage cannot be avoided completely.

The majority of the existing pre-analytical studies on metabolite stability during storage were focused on short-term stability and effects of repeated freeze-thaw cycles on the metabolic fingerprint [10–12,15,21,22]. The influence of long-term storage on the metabolite profile is yet underrepresented in literature. While Yang et al. compared two completely different plasma sample cohorts at two time points within a five-year time frame [14], Hustad et al. modelled the kinetic effects of storage time on plasma metabolite concentrations at five time points between 0 and 27 years [23]. However, the latter study was restricted to 32 compounds related to only the vitamin B metabolism. A much larger amount of metabolites was studied by Abuja et al., but the effect of storage time was only mimicked by repeated changes of the storage temperature [24].

Here, we present the first study that investigates the time-dependent course of the metabolite concentrations of a set of 111 metabolites (acylcarnitines, amino acids, glycerophospholipids, hexoses, and sphingolipids) in a pooled plasma sample that was stored at -80 °C for five years. During this time period, 1012 aliquots of the plasma sample were measured at 193 independent time points with the Biocrates Absolute*IDQ*® p180 targeted-metabolomics assay.

In an interlaboratory ring trial it was demonstrated that this assay allows highly reproducible quantification of most of the analysed metabolites [25], and it has been successfully applied in numerous clinical studies such as KORA [26], EPIC-Potsdam [27], VARIETE [28], POGO/PINGUIN [29], INFOGENE [30], and ENIGI [31].

For the data analysis we used a linear mixed effects regression model to model the metabolite concentration trends and calculated the percentage changes of metabolite concentration levels over time.

MATERIALS AND METHODS

POOLDED PLASMA SAMPLE

A pooled EDTA-plasma of 500 mL from 6 different volunteers (3 male, 3 female, mean age 31.5 years) was bought from Seralab (Sera Laboratories International Ltd, Hull, United Kingdom) in January 2011. The frozen plasma was thawed once upon delivery and aliquoted into 100 µL aliquots using a Hamilton Microlab STAR™ roboting unit (Hamilton Bonaduz AG, Bonaduz, Switzerland). The aliquots were immediately stored at -80 °C. Since that time, the plasma was routinely used as internal control, and reference plasma in our targeted-metabolomics platform.

Each day a metabolomics project was measured with the Biocrates Absolute*IDQ*® p180 kit 96-well plate (Biocrates Life Science AG, Innsbruck, Austria), one aliquot of the same reference plasma was thawed at room temperature and was measured in triplicates to quintuplicates together with the samples of the project. To minimize batch effects, the replicates were always randomly distributed on the 96-well kit plates. In total 1076 aliquots at 197 time points were measured between January 2011 and January 2016.

TARGETED METABOLOMICS ANALYSIS

All metabolites were quantified using the Biocrates Absolute*IDQ*® p180 kit as described in detail previously [32]. The kit allows the quantification of 188 endogenous metabolites from six different compound classes (amino acids, biogenic amino acids, acylcarnitines, glycerophospholipids, sphingolipids, and hexoses), and is validated according to FDA guidelines 'Guidance for industry – Bioanalytical Method Validation (May, 2001)'. Absolute quantification of amino acids and biogenic amines was achieved by using chromatographic separation by liquid

chromatography (LC), isotopically labelled internal standards, and calibration curves. The quantification of acylcarnitines [Cx:y], sphingomyelins [SM Cx:y], hydroxysphingomyelins [SM (OH) Cx:y], lysophosphatidylcholines [lyso Cx:y], phosphatidylcholines [PC z Cx:y] and the sum of hexoses [H1] was based on one or more internal standards for each metabolite class. X and y denote the number of carbon atoms and the number of double bonds, respectively. The two lipid side chains of the phosphatidylcholines can be linked to the glycerophosphatidyl headgroup via an acyl-acyl ester bond (z = aa) or a mixed acyl-alkyl bond (z = ae).

All liquid handling steps were performed with a Hamilton Microlab Star™ robot. Multiple reaction monitoring (MRM) measurements were conducted with a Sciex API4000™ mass spectrometer (Sciex Deutschland GmbH, Darmstadt, Germany) coupled to an Agilent 1200 series HPLC (Agilent Technologies Deutschland GmbH, Böblingen, Germany), and a HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland), controlled by the software Analyst (1.4 to 1.6.1). FIA-ESI-MS/MS analysis (flow injection analysis-electrospray ionisation-mass spectrometry) was used for the analysis of acylcarnitines, phospholipids, sphingolipids, and the sum of hexoses. MRM spectra of amino acids and biogenic amino acids were acquired in a separate LC-ESI-MS/MS run. LC data were primarily analysed with the software packages Analyst and MultiQuant 3.0. The Met*IDQ*® software provided by Biocrates was used for quality assessment, data evaluation, and quantification of the metabolites of the FIA-measurements. Metabolite concentrations are reported in µM.

STATISTICAL DATA ANALYSIS

All data pre-processing and analyses steps were performed using the statistical software R version 3.2.3 [33]. Data quality was ensured in a three step procedure: First, single metabolites were excluded if one or more of the following criteria were met: (i) more than 15% of the 96-well plates had a coefficient of variation greater than 15%; (ii) more than 50% of the measured samples contained missing values; (iii) more than 25% of the measured samples had a metabolite concentration below the limit of detection.

Subsequently, missing values (degree of missingness = 0.3% of total data points) were imputed with the R package "impute" using the k-nearest neighbour method with k = 6 [24,34,35]. In a second step, strong outlier samples were detected via robust principal component analysis (PCA) and Hotelling's T^2 statistics [36,37] using the package *pcaMethods* [38]. Samples outside the 95% confidence interval were regarded as strong outliers and were removed from the data set (25 samples). In a third step, samples with more than 33% of the metabolites exhibiting concentrations outside the ± 1.5 x quantile range were excluded from further analyses (39 samples). After these quality control (QC) steps, 1012 samples, representing 193 measurement time points within a five-year time period and 111 metabolites remained in the data set.

We conducted a PCA on the quality controlled data set with the R package *pcaMethods*, version 1.58.0 [38].

Linear mixed effect models were computed using the R package *nlme*, version 3.1-127 [39]. A random intercept was used for each 96-well plate. We included the variables 'storage days' and 'measured samples since last maintenance' as fixed effects. The latter was used as a proxy for the contamination of the mass spectrometer that inevitably occurs during high-throughput measurements of plasma samples. It was defined as the sum of all samples that were measured

with the Sciex API4000™ mass spectrometer since the last major maintenance event that included a thorough cleaning of all quadrupoles by a technical engineer.

As a measure of concentration change over time, we calculated the percentage change of the difference between the predicted concentration value at the most recent time point (corresponding to 1842 storage days, with 'samples since maintenance' set to zero) and the concentration at storage day zero, reflected by the intercept of the mixed effects model. 95% confidence intervals (BCa percentile) were calculated by computing 2000 bootstrap replicates with the R package *boot*, version 1.3-18 [40,41]. Analytical P-values were confirmed by a permutation test with 10^5 permutations.

RESULTS

Prolonged storage of biospecimen can alter the concentration levels of metabolites during biobanking and thus bias the data analysis. Especially in longitudinal studies, where samples are collected, stored and measured over a time range of years or even decades, this becomes of crucial importance. Therefore, we investigated the influence of storage time on the metabolite concentrations of 111 metabolites in a pooled human plasma that was stored at -80 °C in January 2011. *O*ver a time period of five years, i.e. between January 2011 and January 2016 we successively measured 1076 aliquots of the pooled plasma (1012 aliquots remained after quality control) using the Biocrates Absolute*IDQ*® p180 kit.

After quality control, we conducted a principal component analysis (PCA) to get a first overview on the data set (Figure 1). The PCA scores plot revealed two distinct clusters that are separated by the first principal component. To determine which pre-analytical factors were underlying this clustering, we coloured the samples in Figure 1 according to different aspects, including 'storage time' (1A), 'season' (1B), 'measured samples since last maintenance' (1C),

and 'kit plate delivery batch number' (1D). Figure 1A clearly shows that the samples in one of the clusters were mainly measured in 2012. As illustrated by the loadings, the driving forces separating the two clusters in the first principal component were the phosphatidylcholines (PCs) and the lysophosphatidylcholines (Figure S-1). Indeed, particularly the PCs with carbon numbers between 32 and 36 exhibited strongly increased concentrations in 2012 (Figure S-2). For the same time period strongly decreased concentration levels were observed for the sphingomyelins, which constituted the dominating species in the second principal component.

Strikingly, the concentration levels of most of the glycerophospholipids followed a waveform-like pattern over time indicating between-batch effects (Figure S-2). We first hypothesised a temperature effect triggered by the seasons. However, Figure 1B disapproved the hypothesis since within each cluster samples measured in all seasons can be found.

We argue that the waveform-like pattern was based on pronounced matrix effects whose characteristics were changing periodically by accumulation of contaminations as it alternated directly with thorough routine cleaning of the ion path of the mass spectrometer. This assumption was supported by the fact that in some cases abrupt changes in concentration levels coincided with major maintenance events (see for instance Figure S-2, Page S-81, PC ae C44:6). Furthermore, we observed a tendency that the amplitude of the periodic pattern of an analyte was reciprocal to the chemical similarity to its internal standard. In cases where the chemical structure of an analyte was close to its internal standard (e.g. amino acids, free carnitine, PC aa 40:2) abrupt changes in concentration levels and periodic patterns were almost completely absent.

Basically, the observed clustering of the samples measured in 2012 could have also been caused by the use of different delivery batches of the Biocrates Absolute*IDQ*® Kit 96-well plates.

However, we would exclude a major influence of the Biocrates delivery batches. First, the regression of the Biocrates delivery batch numbers on the principal components did not reveal a significant effect for the first ten principal components (Table S-1). Second, if the Biocrates delivery batches were responsible for the observed clustering, then the analyte concentrations should have remained constant within a delivery batch. However, as can be seen for instance for sphingomyelin SM C24:1 (Figure S−3, Page S-203), the concentrations within the batches are altered gradually (e.g. delivery batch number 3). In some cases where samples were measured on the same delivery batch but with interruption (by other delivery batches used in between), the metabolite concentrations were not constant but on different levels (e.g. Figure S−3, Page S-203, SM C24:1, delivery batch number 3, 7 and 13).

Thus, we conclude that the separation of the two clusters in the PCA is mainly driven by different matrix effects due to different amounts of impurities in the ion path of the mass spectrometer.

To account for the contamination of the mass spectrometer in our model, we introduced the variable ‘measured samples since last maintenance’, which indeed perfectly explains the

separation of the two clusters seen in the PCA (Figure 1C, Table S-1).

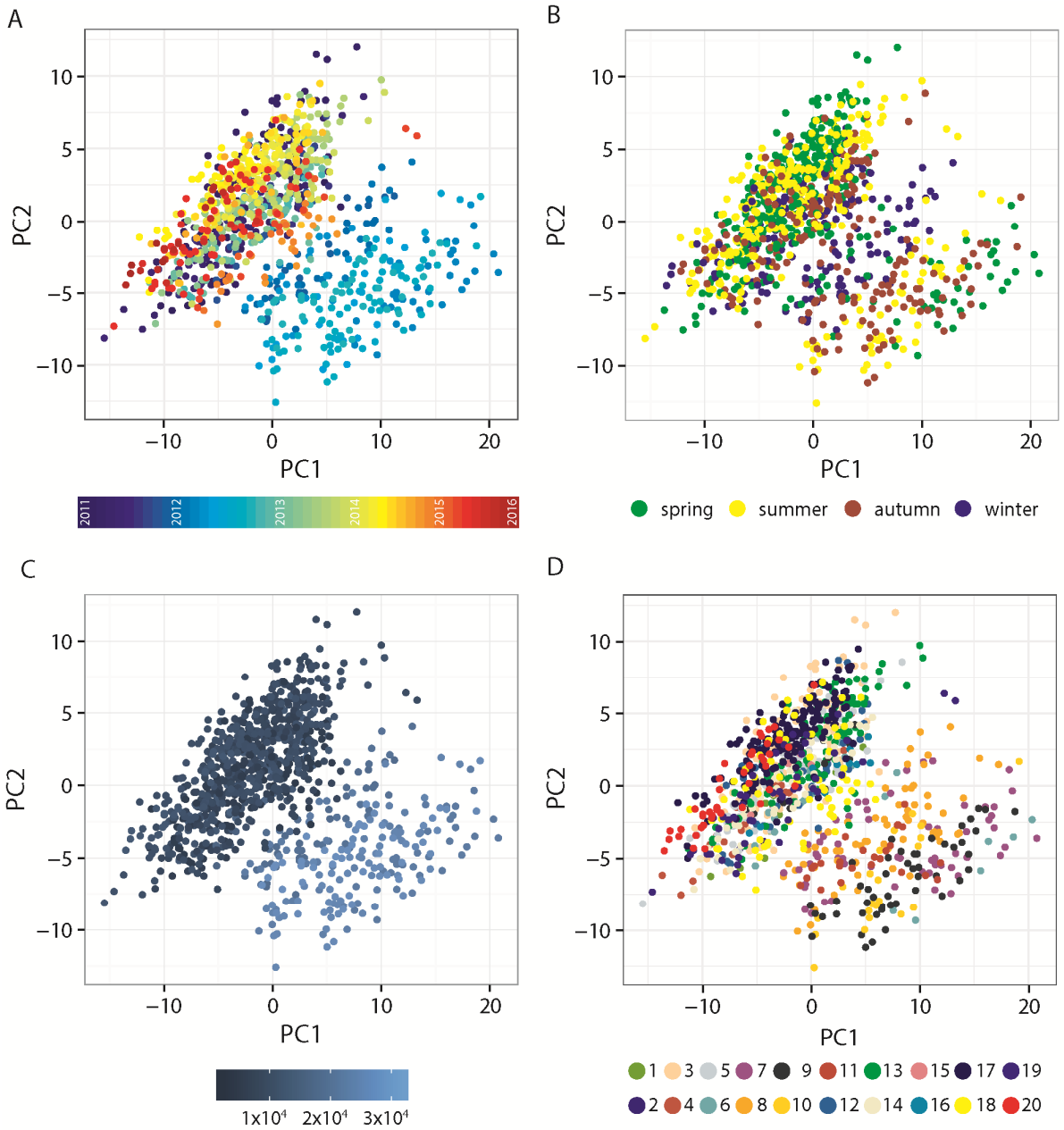

Figure 1. PCA scores plot of the data set. Labelling of data points according to: (A) Time of measurement. (B) Seasons. (C) Number of measured samples since last maintenance event. (D). Delivery batches of the Biocrates Absolute*IDQ* p180 assay. The color scheme for the Biocrates delivery batches is identical to the one used in Figure S-3.

Modelling of the metabolite concentration courses over time

Hustad et al. noted a linear course for the concentration levels of the investigated metabolites within the first five years of their study [23]. Based on this finding and visual inspection of our data, we also hypothesised a linear course of the metabolite concentrations over time. We applied a linear mixed effects model including a random effect for the replicates on each measured 96-well plate. In comparison to a linear model, the mixed effects model led to reduced residuals and considerable reduced autocorrelation (Figure S-4).

To identify stable and unstable metabolites, we tested whether the slopes, i.e. the concentration changes over time, were significantly different from zero (Table S-2). In those cases where the concentration changes were small in comparison to the variance of the data, the P-values were larger than the 5% significance threshold. These metabolites were considered as stable. Metabolite changes were regarded as significantly altered, when the concentration change over time was large in comparison to the variance of the data. To take into account the model uncertainty that is introduced by the observed batch effects we used a bootstrapping procedure with 2000 replicates to calculate confidence intervals for the percent change over time for each metabolite.

Figure 2 shows the changes of metabolite concentrations in the plasma samples after storage for five years at -80 °C. Fifty-five out of 111 metabolites remained stable. For all significantly increased or decreased metabolites the average percentage change was +13.7% (SD = 4.9%) or -14.5% (SD = 1.9%), respectively.

In detail, eight significantly altered amino acids showed elevated concentration levels with a mean increase of 15.4% ± 4.4% (Table 1). Similarly, the level of the sum of hexoses was increased by 7.9%. With the exception of butyrylcarnitine (+9.4%), all significantly altered

carnitine (-12.1% ± 3.5%), sphingomyelin (-14.8% ± 2.4%), and lysophosphatidylcholine (-15.1% ± 3.3%) concentrations were found to be reduced after five years of storage. More heterogeneous alterations were observed for the phosphatidylcholines. Although phosphatidylcholines with a chain length of less than 40 carbon atoms tended to be decreased, those with a chain length above 40 carbon atoms were found to be predominantly increased. An influence of the degree of saturation on the concentration change pattern was not discernible.

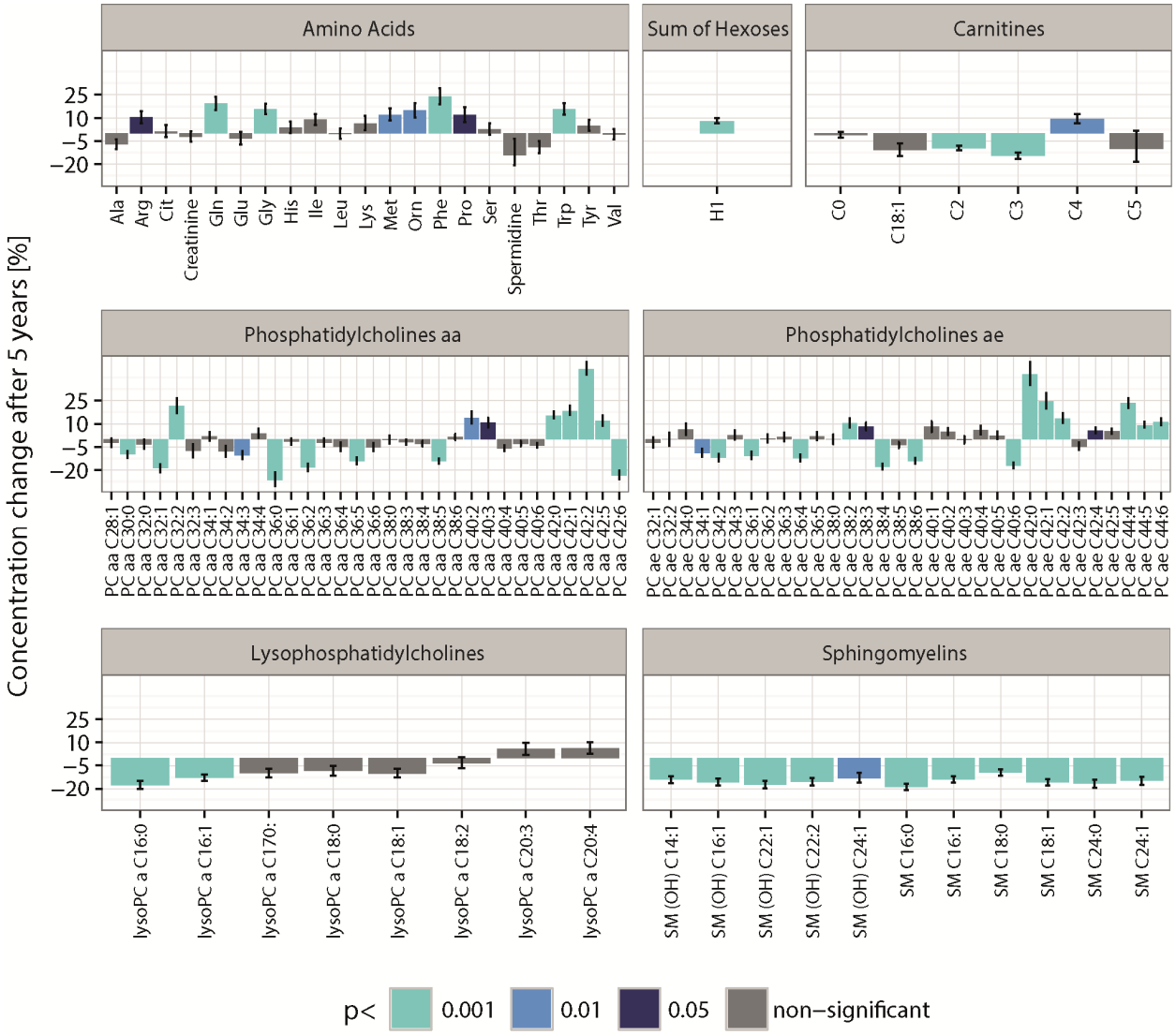

Figure 2. Percentage changes of metabolite concentrations after five years of storage at -80 °C. The error bars indicate 95% confidence intervals based on 2000 bootstrap replicates.

DISCUSSION

We investigated the change of plasma metabolite concentrations during long-term storage in a freezer at -80 °C. In the course of this study, a number of statistically significantly altered metabolites were found. The sources for these changes may be various. Basically, oxidation reactions, acid-base driven hydrolyses, and also enzymatic activities are conceivable. However, all these reactions should be discussed in the light of the freezing process and the physicochemical conditions at low temperatures.

When an aqueous mixture is frozen below the freezing point of water, the water starts to crystallize and the remaining components are concentrated. Due to the concentration process, the melting point of the remaining liquid is further reduced until the eutectic point is reached. At temperatures below the eutectic point a mixture becomes completely solid. Depending on the cooling rate the mixture becomes either crystallized (slow freezing) or vitrified (fast freezing) [42,43]. Interestingly, no clear eutectic point has been observed so far for human plasma when plasma was studied in the range between 0 °C and – 65 °C [42,44]. Thus chemical reactions maybe possible in small micropores or even nanopores [45] at least as long a sample is not completely solidified [46,47].

At temperatures below -65 °C proteins usually undergo a transition where the motion of atom groups stops and as a consequence enzymatic reactions are either extremely slow or non-existent [48]. Dunn and Daniel reported very small residual catalytic activities for catalase and alkaline phosphatase at temperatures down to -100 °C when soluted in cryosolvents [49]. The authors argue that the catalytic activity in these cases is not limited to the motion of atom groups but the availability of the substrate. However, they could not detect any enzymatic activity in frozen mixtures without cryosolvents below -40 °C.

Due to the lack of cryosolvents in the plasma sample investigated here, we would therefore exclude enzymatic activity as an explanation for the observed concentration changes in our study at least for the time the samples were completely frozen. However, sample handling during storage might influence the sample composition. For instance, Abuja et al. observed an increase of the sample core temperature of approximately 40 °C when retrieving a sample from liquid nitrogen (-195 °C) and exposing it to ambient temperature for 90 s [24]. It is thus conceivable for our samples that when the box containing the plasma aliquots was retrieved from the freezer to take out an aliquot for analysis, some of the remaining plasma aliquots were heated to a temperature that allowed formation of a liquid phase at least at a microscale level at the border between plasma and the tube wall. Thus, enzymatic activity may have occurred during the time of warming and refreezing of the respective aliquots.

Beside enzymatic conversion, simple hydrolysis reactions or oxidative processes may have occurred during long term storage. Movement of H^+ and OH^- ions is possible even in solid ice [50,51]. In addition, the formation of ice leads to a concentration increase for the remaining solutes accompanied with a change in pH, solubility, viscosity, and thermodynamic properties [43].. Thus, in our plasma samples acidic or alkaline hydrolysis may have occurred and this may have contributed to the observed metabolite concentration changes.

In proteins, not only the peptide bonds are susceptible to non-enzymatic hydrolysis, but also the side chains of some amino acids. Particularly, asparagine and glutamine are converted to their dicarboxylic acid counterparts aspartate and glutamate by deamidation [52]. However, the significant increase of glutamine in our aliquots showed that a hypothetic deamidation process was either absent or much slower than the release of amino acids from the proteins by hydrolysis of peptide bonds. Augmented levels of amino acids during storage were also observed in other

studies [12,21,23,53]. Congruent with the long-term study of Hustad et al. [23] , we observed increased concentration levels of arginine, glycine, and serine. On the other hand, we did not detect decreased concentrations of methionine or significantly elevated levels of its oxidation product methionine sulfoxide. The reasons for this discrepancy may be various. First, the study by Hustad *et al.* implies a large biological range of the methionine level within the investigated population consisting of 130 samples per time point. As we have investigated a single pooled sample of five people only, our methionine result may not be representative. Second, the samples in the study by Hustad *et al.* were stored at -25 °C only. According to the Arrhenius law, reaction kinetics strongly depends on temperature. Thus, it can be assumed that oxidation reactions may be more pronounced at -25 °C than at -80 °C.

That oxidation processes played only a minor role in our study is also indicated by the increase of the tryptophan concentration. As the indole ring system of this amino acid is prone to oxidation reactions [54], a decrease of the tryptophan levels would have been expected in case of pronounced oxidation.

The accumulation of hexose species on long-term storage can be explained by the release of sugar moieties from glycosylated proteins (e.g. HbA1c, globulins) and glycosylated lipids, particularly from lipoprotein particles (LDL, HDL, VDL) due to hydrolysis of the glycosidic bonds [55].

In contrast to amino acids and hexoses, whose accumulation could be explained by the decay of larger entities, most of the investigated lipids and acylcarnitines were depleted over time. The reduced concentrations of acylcarnitines were presumably a consequence of the hydrolysis of the ester bonds between carnitine and fatty acids.

Hustad et al. noted an accumulation of free choline at a rate of 8.68% per year with an overall change of 260% after 27 years of storage at -25 °C [23]. Although a residual activity of enzymes (e.g. esterases) is conceivable at this temperature, the choline headgroup appears to be susceptible to hydrolysis. This would explain the tendency of reduced levels of sphingomyelins, phosphatidylcholines, and lysophosphatidylcholines in our long term stored samples. Another plausible explanation for the detection of reduced lipid ester levels would be the (per)oxidation of the fatty acid double bonds [56,57]. However, reduced concentration levels were also found for saturated sphingolipids and glycerophospholipids and we did not observe a degree of saturation dependent depletion of choline-containing lipids Again, we conclude that oxidative processes played a minor role in our study.

In contrast to our findings, Yang et al. reported an increase of lysophosphatidylcholines after five years of storage at -80 °C and explained this by the activity of the lecithin-cholesterol acyltransferase (LCAT) [58]. But these results have to be considered with caution because they have compared two completely different cohorts using a semi-quantitative approach. Both cohorts were sampled with a time difference of five years. Thus, the observed changes may just reflect the differences between the two different cohorts or some differences during the sampling procedure.

That sample handling can have an influence on metabolomics result data can be learned from a study of Anton et al. who have shown that a high ratio of lysophosphatidylcholines to phosphatidylcholines indicates prolonged storage of plasma samples at room temperature [12]. The authors suggest to use this specific metabolite ratio to assess sample quality [12]. With regard to the phosphatidylcholines with chain lengths above 40 carbon atoms, some technical aspects of the targeted metabolomics approach have to be taken into consideration. Contrary to the

phosphatidylcholines with lower chain lengths, those long chain lipids exhibited predominantly elevated concentrations in our samples after 5 years (Fig 2). Particularly, the concentration courses of PC aa C42:x, PC ae C42:x, and PC ae C44:x exhibited abrupt and pronounced changes during the investigated time period, which made it difficult to model the data correctly (Figure S-2, Page S-45 *ff*., Page S-80 *ff*.). Apparently, the measurements of these metabolites are very prone to technical disturbances like contamination of the ion path of the mass spectrometer. As the waveform-like pattern of the concentration courses showed, measurements of the glycerophospholipids and sphingolipids were generally more perturbed than those of the amino acids or acylcarnitines and thus less reliable. Presumably, this was caused by pronounced matrix effects in the flow injection mode and a far lower density of internal standards used for the quantification of these metabolites classes. Furthermore, we cannot exclude that at least some of the observed effects for these metabolite classes are a consequence of the repeated retrieval of the aliquot box from the freezer that may have led to microscale thawing with subsequent hydrolytic or enzymatic activity. However, Abuja et al. have shown that the concentration change was below 2.5% for almost all investigated metabolites after 1000 freeze-thaw cycles between -80 °C and +20 °C [24]. It is also important to note that our experiment is based on a single pooled plasma sample and from young donors (mean = 31.5 years) only. The behavior of metabolites observed in this plasma matrix might not be representative for all cases, i.e. concentration changes observed in our plasma sample could be different in plasma of other origins, for instance plasma of obese probands.

In our study, we have investigated the stability of 111 human plasma metabolites from six different metabolite classes during storage at -80 °C over a time period of five years. For fifty-five of these metabolites (50%) we observed statistically significant changes. Looking at the

metabolite classes, the levels of amino acids and the sum of hexoses were either stable or elevated, the concentrations of carnitines, phosphatidylcholines and sphingomyelins showed a tendency to be either stable or decreased. The mean increase and decrease of the statistically significant altered metabolites was +13.7% and -14.4%, respectively.

However, our study is limited to a single pooled plasma sample only, and albeit cautious modelling our results might be biased. Thus, a replication study using multiple plasma samples would be desirable.

The observed concentration changes are presumably based on hydrolysis reactions but also enzymatic activity during retrieval of the aliquots from the freezer may be conceivable. We haven't found evidence for pronounced oxidative processes.

We conclude that long-term storage of human plasma samples even at -80 °C can lead to statistically significant changes of metabolite concentrations.

Based on our findings, several recommendations with regard to sample handling/storage, choice of the analytical platform, and the global study design can be deduced:

1. Ideally, plasma samples should be snap-frozen and stored in liquid nitrogen immediately after centrifugation of the blood. The fast cooling rate and the low temperature allows vitrification of the mixture and thus any possible chemical reactions that could alter the plasma metabolome should be absent or at least extremely slow.
2. For mass spectrometry based high-throughput metabolomics platforms it is vitally important to schedule regular service intervals that include thorough cleaning of the ion path of the mass spectrometer. Otherwise, accumulation of particles on the quadrupoles may hinder accurate determination of metabolite concentrations. By using multiple QC

samples at one or more concentration levels that are analysed together with the samples under investigation the status of the mass spectrometer can be monitored.

3. In longitudinal studies, where the collection of baseline and follow-up samples lead to different storage times, the deterioration of plasma samples can significantly bias the outcome of the data analysis [59]. By regularly measuring aliquots of a pooled QC sample containing the metabolites under investigation, a regression model can be deduced and applied to account for the storage effects. Ideally, this QC sample should be a pooled sample consisting of aliquots of all study samples. In cases where this might not feasible, for instance due to lack of sufficient sample volume, a pooled sample consisting of the same matrix as the samples under investigation should be used.

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ASSOCIATED CONTENT

Supplemental Figure S-1. Contribution of PCA loadings to the principal components one and two

Supplemental Figure S-2. Time-dependent concentration courses of 111 human plasma metabolites including the regression lines. The arrows indicate major maintenance events.

Supplemental Figure S-3. Time-dependent concentration courses of 111 human plasma metabolites including 96-well plate delivery batches of the Biocrates Absolute*IDQ*® p180 Kit. Each colour represents a delivery batch. The arrows indicate major maintenance events. The colour code scheme and the measurement sequence of the Biocrates delivery batches are presented on page S-115.

Supplemental Figure S-4. Residual and autocorrelation plots of 111 human plasma metabolites. The results are plotted for the linear regression model (upper row) and the linear mixed effects regression model (lower row). The dotted blue lines in the autocorrelation plots (ACF = auto correlation function) represent the significance threshold for autocorrelation within the residuals.

Supplemental Table S-1. P-values for regression of different covariates on PCA scores of the first ten principal components.

Supplemental Table S-2. Regression parameters of linear mixed effects model and percent change of metabolite concentrations after storage at -80°C for five years. The dimension of slopes is μM x day^{-1}.

Supplemental Table S-3. Quality control of Biocrates Absolute*IDQ*™ p180 metabolites

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. All authors declare no competing financial interests.

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ABBREVIATIONS

FDA, U.S. Food & Drug Administration; SM, Sphingomyelin; SM (OH), Hydroxysphingomyelin; PC aa, Phosphatidylcholine acyl-acyl;PC ae, Phosphatidylcholine acyl-alkyl; MRM, Multiple Reaction Monitoring; FIA-ESI-MS/MS, Flow injection analysis electrospray ionisation tandem mass spectrometry; LC-ESI-MS/MS, Liquid chromatography electrospray ionisation tandem mass spectrometry; PCA, Principal component analysis; PCs, Phosphatidylcholines; HbA1c, Haemoglobin A1c (glycated Haemoglobin A1); LDL, Low-density lipoprotein particles; HDL, High-density lipoprotein particles; VDL, Very low-density lipoprotein particles; LCAT, Lecithin-cholesterol acyltransferase. ACF, Autocorrelation function

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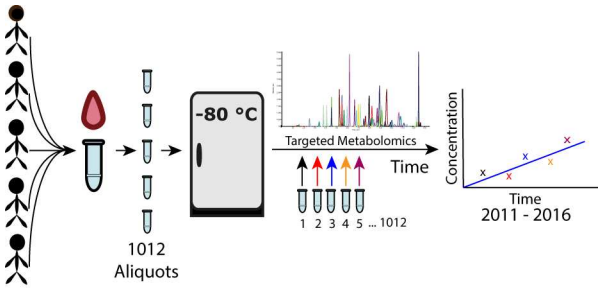
TABLES.

Table 1. Number of stable/unstable metabolites per metabolite class and mean percentage change of the significantly altered metabolites**.**

Metabolite Class	Number of stable metabolites (non-significantly changed)	Number of unstable metabolites (significantly changed)	Mean increase of significantly changed metabolites [%] (SD)	Mean decrease of significantly changed metabolites [%] (SD)
Amino Acids	13	8	15.4 (4.4)	-
Carnitines	3	3	9.4	-12.1 (3.5)
Phosphatidylcholines aa	17	15	19.6 (12.0)	-17.0 (5.9)
Phosphatidylcholines ae	16	16	16.4 (11.6)	-13.3 (3.3)
Lysophosphatidylcholines	6	2	-	-15.1 (3.3)
Sphingomyelins	0	11	-	-14.8 (2.4)
Sum of Hexoses	0	1	7.9	-
Overall	55	56	13.7	-14.5

Graphical Abstract



for TOC only

Figure Legends

Figure 1. PCA scores plot of the data set. Labelling of data points according to: (A) Time of measurement. (B) Seasons. (C) Number of measured samples since last maintenance event. (D). Delivery batches of the Biocrates Absolute*IDQ* p180 kit. The color scheme for the Biocrates delivery batches is identical to the one used in Figure S-3.

Figure 2. Percentage changes of metabolite concentrations after five years of storage at -80 °C. The error bars indicate (bootstrapped) 95% confidence intervals.

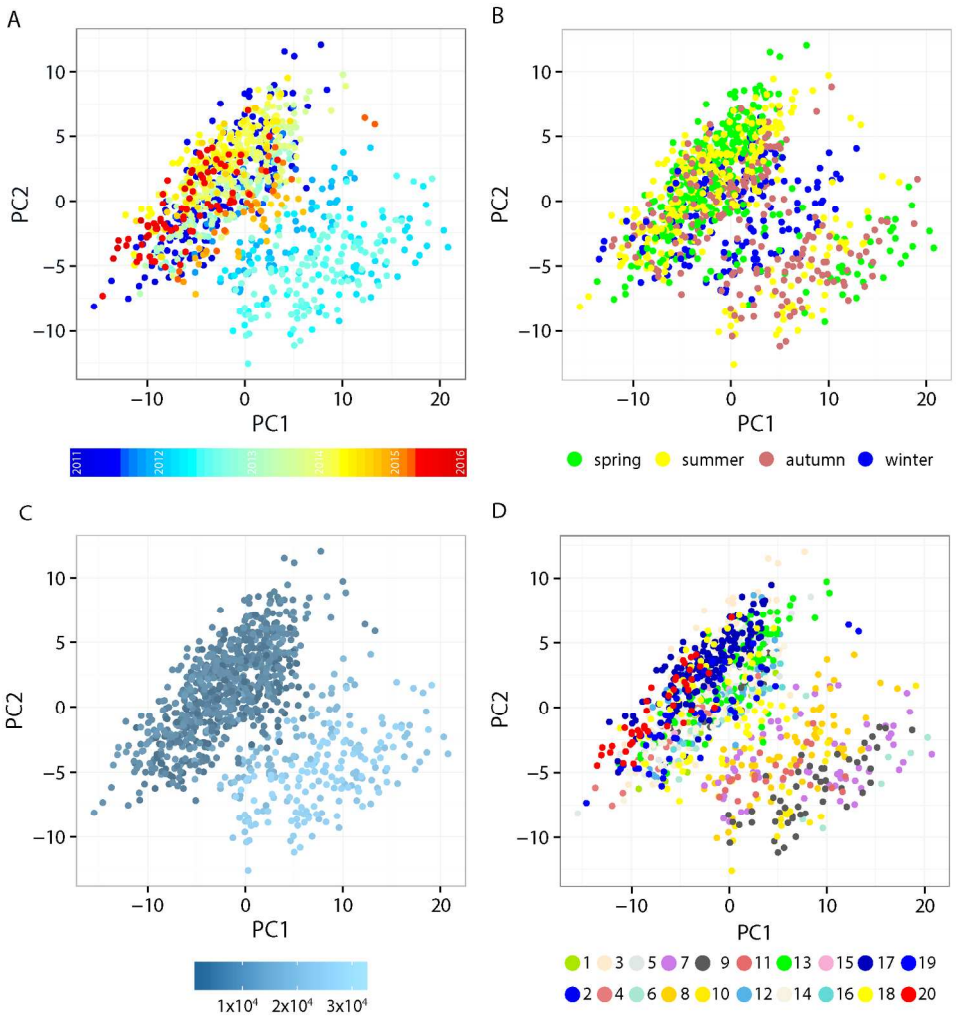


Figure 1. PCA scores plot of the data set. Labelling of data points according to: (A) Time of measurement. (B) Seasons. (C) Number of measured samples since last maintenance event. (D). Production batches of the Biocrates AbsoluteIDQ p180 assay. The color scheme for the Biocrates production batches is identical to the one used in Figure S-3.

176x178mm (300 x 300 DPI)

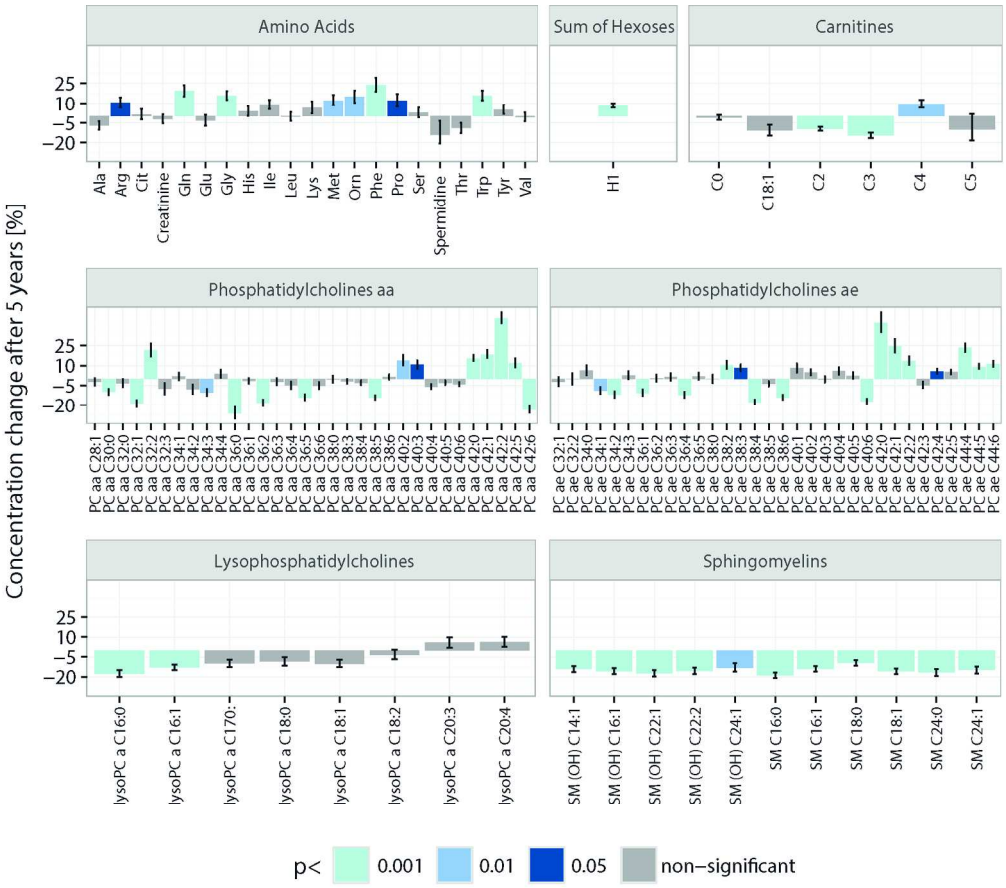
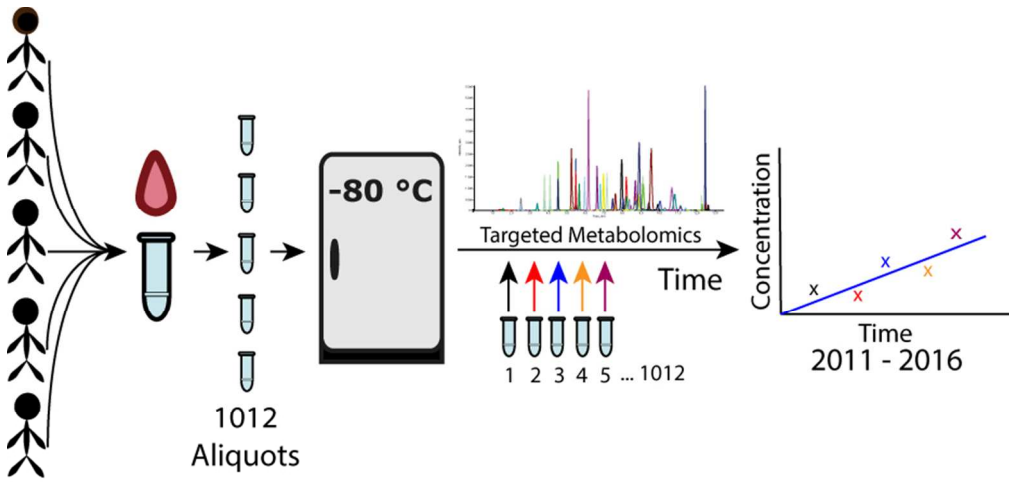


Figure 2. Percentage changes of metabolite concentrations after five years of storage at -80 °C. The error bars indicate (bootstrapped) 95% confidence intervals.

172x154mm (300 x 300 DPI)



for TOC only

78x39mm (300 x 300 DPI)