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Human plasma stability during handling and storage: impact on NMR metabolomics

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Abstract

This work contributes to fill in some existing gaps in the knowledge of human plasma degradability during handling and storage, a paramount issue in Nuclear Magnetic Resonance (NMR) metabolomics. Regarding the comparison between heparin and EDTA anti-coagulant collection tubes, the former showed no interference of the polysaccharide, while conserving full spectral information. In relation to time/temperature conditions, room temperature was seen to have a large impact on lipoproteins and choline compounds from 2.5 hours. In addition, short-term storage at -20°C was found suitable up to 7 days but, for periods up to 1 month, -80°C was recommended. Furthermore, in the case of reusing plasma samples, no more than 3 consecutive freeze-thaw cycles were found advisable. Finally, the impact of long-term -80°C storage (up to 2.5 years) was found almost negligible, as evaluated on a partially matched non-fasting cohort (n=49), after having investigated the possible confounding nature of the particular non-fasting conditions employed.

Keywords

Human plasma, metabolomics, metabonomics, heparin, EDTA, anti-coagulant, stability, storage, confounders, fasting

1. INTRODUCTION

Metabolomics has emerged as an invaluable tool in disease research[1,2], based on the analysis of biofluids and biological tissues by analytical strategies with the ability to detect wide ranges of metabolites, in a relatively short time. Such strategies are typically based on Nuclear Magnetic Resonance (NMR) spectroscopy or Mass Spectrometry (MS) detection (coupled to chromatographic methods). These have been increasingly employed as complementary windows to the same problem, thus exploiting their different characteristics (sensitivity, reproducibility, specifics of analysis: targeted or untargeted) towards improved compound detection and resolution. In typical metabolomic studies, both NMR and MS datasets of biological complex samples are usually vast, requiring the use of data mining methodologies based on multivariate analysis (MVA) for their handling and interpretation in terms of potential metabolite biomarkers. In disease research, blood plasma and serum metabolomics is extremely useful since it reflects the circulating metabolome of the organism, thus providing an average fingerprint of the biochemical status of the organism and of deviations from homeostasis. However, much concern has been expressed in relation to the high perishability of blood plasma and serum[3–5] and its impact on the detectable metabolome, viewed both by NMR and MS. It has been recognised that the implementation of adequate intra- and inter-laboratory standard operating procedures (SOPs) for blood samples is imperative and several possible bias sources have been identified: (i) blood plasma collection tubes containing different anti-coagulants, namely ethylene diamine tetraacetic acid (EDTA), citrate and heparin, (ii) different temperature/time conditions during sample handling, analysis and storage and (iii) multiple freeze-thaw cycles, when it is necessary to reuse the sample for confirmation or analysis by complementary methods (e.g. MS, metabolite extraction, genetic, proteomic assays). However, in spite

of the high work volume carried out in this context, a few questions remain unanswered thus justifying the present paper.

In the case of the effects of different anticoagulant-containing tubes, most studies have been performed by MS methods, which have mostly recommended the use of EDTA tubes[4,6–9], although recent reports point at some advantages of the use of heparin tubes[10,11]. In terms of the effects viewed by ^{1}H NMR spectra, only one report exists to our knowledge, considering EDTA and citrate tubes (but not heparin) for human plasma collection[12]. The authors claim that no significant interaction is seen to occur between anti-coagulants and endogenous compounds and that some of the information overlapped with anti-coagulant peaks may be recovered to some extent (e.g. through *J*-resolved NMR spectroscopy). In the present work, the effects of EDTA and heparin collection tubes on human plasma composition are compared by NMR spectroscopy.

In relation to temperature/time conditions during handling, analysis or storage, a significant number of studies have been carried out by both MS[6,7,9,11,13] and NMR[3,14-16]. Regarding NMR studies, the effects of room temperature stability were studied for 1-3h (human plasma), 15 and 24 h (rat plasma)[3,14]. Studies of refrigeration/freezing conditions have been reported at a) 4^{o}C for 0, 24, 36 h (human serum) and 1 week (rat plasma); b) -20^{o}C for 1 month (rat plasma) and 2-15 years (bovine plasma); and c) -80^{o}C for 48h, 3, 6 and 9 months (rat plasma)[14–16]. Comparison between the above studies should, however, be carried out with care since samples from different species are known to have distinct compositions (mainly in terms of lipoproteins)[17] and possible different degrees of enzymatic activity and inhibition[18]. Room temperature (RT) stability within a few hours is indeed particularly relevant for consideration of total sample handling time, as well as in the case of non-refrigerated automated sample changing, therefore justifying a thorough hourly study of human blood plasma, as

reported here. In addition, a practical hindrance that may arise in most hospitals or collection centres is that -20°C freezing is often necessary for a few days after collection, before sample transfer to -80°C is possible. Therefore, human plasma stability at -20°C within 30 days is monitored here, in parallel to comparable timings at -80°C. Furthermore, the possible reuse of the plasma sample introduces the need for freeze-thaw (FT) cycles and, indeed, reports have referred the effects of 1, 5 and 10 FT cycles (human serum)[3,19]. Here, we complement such studies by investigating the effects of 1-5 FT cycles on human plasma, since < 5 FT cycles may be a more probable requirement.

Besides the collection and handling considerations discussed above, potential confounders are expected to have important impacts on the blood metabolome e.g. gender[20], age[20,21], body mass index (BMI)[20], diet and fasting/non-fasting[22–24], ethnicity[20], co-morbidities or medication. Hence, subject matching is desirable as far as possible, particularly if an isolated metabolic fingerprint of the disease is sought. However, the value of using real (unmatched or partially matched) populations is also evident for instance when immediate clinical applications are envisaged (e.g. metabolic assays applicable to any patient at any time) or when sample biobanks are available and a number of confounders (including long-term storage effects) must be acknowledged. Our contribution to this complex issue is to address the effects of fasting/non-fasting conditions and long-term -80°C storage on the plasma of a partially matched population. Therefore, in this paper we report a human plasma NMR metabolomics study which evaluates the changes in metabolic profile in connection to a) the use of heparin collection tubes, in comparison to EDTA tubes; b) hourly stability at room temperature up to 21 h, with and without sodium azide as preservative; sample stability c) at -20°C and -80°C up to one month and d) during 5 consecutive FT cycles. Additionally, the

possible confounding effects of non-fasting conditions (2 hours after uncontrolled meal) and of long-term storage at -80°C (up to 2.5 years) were evaluated. This work contributes towards a more complete picture of SOPs for NMR metabolomics of human plasma, while providing information about two potentially important confounders (non-fasting conditions at collection and long-term -80°C storage) in a large partially-matched population.

2. EXPERIMENTAL

2.1 Sample collection and preparation

Table 1 lists the groups of subjects employed in this work, which include, for some studies, healthy pregnant women in their second trimester of pregnancy (this cohort being part of an on-going study in which a large number of samples was available). Such samples do exhibit small compositional differences, compared to those from non-pregnant subjects (in particular, higher lipid levels), however, their stability properties are expected to be at least qualitatively equivalent. All samples were collected at the Maternity Bissaya Barreto, Coimbra (including samples from non-pregnant subjects), under the approval of the ethical committee of the Hospital Center of Coimbra (Refs.18/04 and 29/09) and informed consents were obtained from each participating subject.

Whole blood was collected into sodium heparin tubes (9 mL) for all studies, and also into EDTA (6 mL) tubes for the anti-coagulant comparison study. Upon collection, the samples were centrifuged (1500 x g, 4 °C, 10 min) not more than 30 min after collection. Supernatants were frozen at -20ºC for up to 2 hours and then stored either at -20°C or at – 80ºC. For the short-term stability study (at -20°C and -80°C), samples were split into aliquots prior to freezing. For the FT cycles study, each cycle involved

143 sample thawing at room temperature for 30 min, followed by refreezing at -80°C.
144 Before analysis, samples were thawed at room temperature (for *ca.* 30 min) and 400 µL
145 of saline solution (NaCl 0.9% in 10% D_2O, with 3mM NaN_3 for part of the room
146 temperature study) were added to 200 µL of plasma. The mixtures were centrifuged
147 (4500 x g, 25ºC, 5 min) and transferred into 5mm NMR tubes.

148 **2.2 NMR spectroscopy**

149 NMR spectra were recorded on a Bruker Avance DRX 500 spectrometer equipped with
150 an actively shielded gradient unit with a maximum gradient strength output of 53.5
151 G/cm, at 300 K. For each blood plasma sample, three 1D ^1H NMR spectra were
152 obtained: a standard spectrum, a Carr-Purcell-Meiboom-Gill (CPMG or T_2-edited)
153 spectrum and a diffusion-edited spectrum. Standard spectra were acquired using a
154 noesy1D pulse sequence with tm = 100 ms, a fixed 3 µs t1 delay and water suppression
155 during relaxation delay (4 s) and mixing time. CPMG spectra were acquired with the
156 RD-90°-{τ-180°- τ}n-acquire pulse sequence, with water presaturation, n= 80, τ = 400
157 µs and a total spin-spin relaxation time (2nτ) of 64 ms. Diffusion-edited spectra were
158 recorded using the bipolar pulse longitudinal eddy current delay (BPPLED) pulse
159 sequence, using sine gradients with 2 ms duration, 90% of the maximum gradient
160 strength (48.15 G/cm) and a 100 ms diffusion time. All plasma 1D spectra were
161 acquired with 32k complex data points, 10330.58 Hz spectral width (SW) and 4s
162 relaxation delay with 1.59 s of acquisition time. Each free induction decay (FID) was
163 zero-filled to 64k points and multiplied by a 0.3Hz exponential line-broadening function
164 prior to Fourier transformation. Spectra were manually phased and baseline corrected
165 and chemical shifts referenced internally to α-glucose H1 resonance (at δ=5.23 ppm).
166 All peak assignments were carried out with basis on 2D NMR experiments
167 (homonuclear and heteronuclear correlation experiments) and consultation of the Bruker

Biorefcode spectral database, as well as of other existing databases[25,26] and specific compound standard solutions.

2.3 Statistical analysis

Each set of spectra (standard, CPMG and diffusion-edited) was used to construct data matrices for analysis using the full resolution spectra. The water resonance region was excluded, as well as those of ethanol (1.15-1.20 and 3.62-3.68 ppm), which was found randomly in blood samples, possibly due to pre-collection skin disinfection. All spectra were aligned using recursive segment-wise peak alignment[27]to minimize chemical shift variations, and data were normalized through probabilistic quotient normalization (PQN)[28], to account for different sample dilutions. Principal component analysis (PCA)[29] and partial least squares discriminant analysis (PLS-DA)[30] were performed on data scaled by different methods, for comparison purposes (unit variance (UV), Pareto, centered scaling, the latter having been chosen for results shown), using SIMCA-P 11.5 (Umetrics, Umeå, Sweden) software. PCA and PLS-DA loadings were back-transformed according to each scaling method and colored according to each variable importance to the projection (VIP), using Matlab 7.12.0. For PLS-DA models, Monte Carlo cross-validation (MCCV) (7 blocks, 500 runs) was carried out with recovery of Q^2 values and computation of classification rates, specificity and sensitivity[31,32]. In some cases, differences between data sets were determined by subtraction of the PQN normalized spectra. Relevant peaks identified in loadings profiles and difference spectra were integrated in Amix 3.9.5, BrukerBioSpin, Rheinstetten, Germany, and analysed using univariate analysis tests: Shapiro-Wilk normality test, Student's t-test or Wilcoxon test, with R-statistical software[33]. Unidimensional Statistical total correlation spectroscopy (STOCSY)[34] was performed in Matlab 7.12.0 for assignment and search of metabolic correlations in the study of room temperature stability. In the same study,

the coefficient of variation (CV) was computed for each variable (PQN normalized data point) as the ratio between the standard deviation and the mean value; in this calculation, the noise was estimated and removed from the data according to the exclusion criteria if $(y_i^{max} - y_i^{min}) < 3P_{10}(\sigma)$, where y_i^{max} and y_i^{min} are respectively the maximum and minimum intensities for each data point and $P_{10}(\sigma)$ is the 10^{th} percentile of the standard deviation for all data points, based on references 35 and 36.

3. Results and Discussion

3.1 Effects of heparin and EDTA tubes on plasma metabolome

Although EDTA and citrate plasma collection tubes have previously been compared in terms of their impact on the ^{1}H NMR spectra of plasma[12], to our knowledge, heparin tubes have not been investigated in this context, a general assumption being that heparin (a linear polysaccharide composed of sulphated GlcN, GlcNAc, GlcA and IdoA[37,38]) does not add significant broad components to the spectra. Indeed, visual comparison of the standard ^{1}H NMR spectra of plasma collected in EDTA and heparin tubes (overlaid in Figure 1a) shows almost total superposition of all regions of the spectra, except for the region where the predominant EDTA forms resonate (free and complexed with Ca^{2+}/Mg^{2+}). Multivariate analysis of the spectra obtained for five different controls was performed, after removing the spectral regions where EDTA peaks resonate. PCA of spectra of sample pairs (Figure 1b) confirmed the agreement between EDTA- and heparin-collected spectra, the slight deviations noted being smaller than inter-individual variability (which seemed largely determined by lipid content and BMI value, both higher for subject 2). This was confirmed by similar analysis of the CPMG and diffusion-edited spectra and subtraction of heparin- and EDTA-collected sample spectra (not shown). Spectral subtraction unveiled higher levels of pyruvate (26.6 ± 4.7%,

singlet at 2.36 ppm, p=0.0023) and two unassigned compounds (111.9 ± 5.7%, singlet at 2.64 ppm, p=0.0079 and 121.1 ± 12.1%, singlet at 3.31 ppm, p=0.0043) in EDTA-collected samples. Pyruvate was indeed detected in a concentrated blank solution left in an EDTA tube (for 20-30 min), together with acetate (1.91 ppm, s), formate (8.45 ppm, s), lactate (1.33 ppm, d) and several other unidentified small peaks (Figure S1a). Regarding heparin tubes, the corresponding concentrated blank solution spectrum (Figure S1b) showed a clear broad polysaccharide profile, together with a lesser number of interfering resonances (residual formate, lactate and two unidentified signals). For both tube types, however, the expected contaminants concentration should be negligible compared to the plasma spectrum (note that the blank solutions are 10x and 15x more concentrated than a typical plasma sample, Figure S1). Due to the overlap with EDTA peaks, we could not confirm the higher choline levels (up to 10%) noted in a previous targeted LC-MS/MS study[6] for heparin-collected samples, compared to EDTA ones. Given the above results, both EDTA and heparin tubes seem equally suitable for plasma collection for NMR analysis, as long as the loss of sample resonances overlapping with EDTA peaks is affordable. Overlapped metabolites identified here comprised choline, citrate, dimethylamine, glucose, glycerol, *myo*-inositol, His, Met, Thr, Tyr and Val. Of these, citrate, free choline and dimethylamine do not give rise to other resonances and choline-containing compounds such as phosphatidylcholine (PC), lyso-PC and sphingomyelin (SM) are not easily studied in other spectral regions such as the aliphatic or glyceryl regions. A comprehensive list of possible plasma metabolites overlapping with EDTA resonances may be found elsewhere[12].

3.2 Short-term plasma stability at room temperature

Considering either the standard or edited (CPMG and diffusion-edited) ^{1}H NMR spectra, the effects of time at RT on plasma composition became clear roughly from 2.5 h onwards, as shown below. PCA of the CPMG ^{1}H NMR spectra of three independent plasma samples recorded at RT, approximately every hour up to 21 hours, showed that samples follow similar variation trends (Figure 2a). It is noted that the plasma of subject 7, incidentally richer in lipids, seems to exhibit a higher magnitude of variation. Computation of the coefficient of variation (Figure 2b) indicated that the most significant changes (marked red/orange) affect lipid and choline resonances (see spectral expansions in Figure S2). Based on this, several resonances were integrated and plotted in a heatmap (Figure 2c). This showed that some aliphatic lipid resonances were decreased from *ca.* 2.5 h, including LDL/VLDL $(CH_2)_n$ and CH_3 groups at 1.270 and 0.875 ppm[39], respectively. On the other hand, HDL methyls, at 0.850-0.820 ppm[39], were significantly increased, reflecting a *ca.* 6% increase in the intensity ratio [CH_3 HDL]/[CH_3 LDL+VLDL]. In addition, several choline resonances were noted to increase, namely the overlapped contributions of PC, LPC, SM[40] at 3.210 ppm and the peak at 3.194 ppm. We suggest that the increase in choline phospholipids may be related to the HDL/(LDL+VLDL) increase, considering the possible discarding of phospholipids from LDL+VLDL (which, taken together, comprise higher phospholipid content) to form HDL. The peak at 3.194 ppm, assigned here mainly to free choline (since it is intensified in CPMG spectra and almost absent in diffusion-edited spectra), showed an increase of up to 20% with time at room temperature. This is in broad agreement with a previous LC-MS/MS report of increase of choline in human plasma during the first hour at room temperature (*ca.* 10%), reaching up to 30% at > 250 min[6], due to enzymatic cleavage of choline esters. This process seems to occur concomitantly

266 with the lipoprotein changes, as shown by a 1D STOCSY experiment on the free
267 choline resonance (Figure 3a). This confirmed that choline seems to be biochemically
268 correlated to HDL and phospholipids (positive correlation) and to LDL/VLDL (negative
269 correlation). The STOCSY results also helped to identify cleaner and more resolved
270 spin systems for each lipoprotein type: HDL (CH_3: 0.820, 0.840, 0.850; $(CH_2)_n$: 1.220,
271 1.245; other CH_2: 1.52, 1.98, 2.73; CH=CH: 5.28 ppm) and LDL/VLDL (CH_3: 0.875;
272 $(CH_2)_n$: 1.270; other CH_2: 1.57, 2.01, 2.22; CH=CH: 5.30, 5.33 ppm). In this way, two
273 main HC=CH environments were identified as referring to HDL (5.28 ppm) and
274 LDL/VLDL (5.30 ppm) and, thus, varying in opposite directions with the time at RT
275 (Figure 3b). The inverse prominence of these resonances in the CPMG and diffusion-
276 edited spectra (Figure 3b) suggested higher mobility of LDL+VLDL unsaturated lipids,
277 compared to those in HDL. Since the average unsaturation degree given by the area
278 ratio [all HC=CH]/[all CH_3] remains unchanged, a conservative change of fatty acid
279 chains from LDL/VLDL to HDL environments seems to be taking place, without lipid
280 oxidation occurring. As to the origin of the above changes, the RT study carried out
281 with sodium azide produced identical changes (not shown) as in its absence, indicating
282 the occurrence of enzymatic lipolytic action without microbial growth (also confirmed
283 by the absence of other indicators of microbial growth e.g. lactate). Previous studies
284 have indeed reported changes in lipids after 3 hours[3] and 6 hours[5] at RT, however in not
285 as much detail as in this study. Other changes reported in relation to amino acids,
286 glycerol and citrate[3,5,14] were not noted here.
287
288 **3.3 Short-term plasma stability at -20°C and -80°C and effect of freeze-thaw cycles**
289 Regarding the compositional stability of plasma stored up to 1 month at -20°C and at -
290 80°C, both standard and diffusion-edited spectra showed remarkable spectral agreement

over the whole period (not shown), only the CPMG spectra reflecting the occurrence of some variation expressed in PC2 (explaining 6.7% of variability), as shown by PCA (Figure 4a). Visual spectral inspection and integration confirmed changes becoming statistically relevant at 31 days (Figure S3): 1) proline (*ca.* +31%, p=0.033, at -20^{o}C), 2) glucose (*ca.* +9%, p=0.019, at -20^{o}C) and 3) unassigned broad peak at 6.8-7.1 ppm (*ca.* -50%, p=0.00036 and 0.0022, at -20^{o}C and -80^{o}C respectively). The significant latter variation (probably due to protein precipitation, also supported by visual observation of the samples) justifies the proximity of samples kept for 1 month at -20^{o}C and -80^{o}C, in the PCA plot in Figure 4a. In previous studies, no significant changes had been seen in the NMR spectra of rat plasma stored for 1 month at -20^{o}C or -80^{o}C[14].

Regarding the effects of up to 5 FT cycles on plasma composition, the PCA of standard spectra shown in Figure 4b expresses an interesting result, since the degree of sample dispersion resulting from FT cycles 1 to 5 is clearly sample dependent. The original plasma composition of subject 10 comprised higher contents of lipids (CH_3, $(CH_2)_n$), choline-containing compounds, Val , Ala and lactate, compared to the remaining two samples, such composition (particularly in regard to lipids) possibly leading to higher degradability of the sample. For this subject, changes upon 5 consecutive FT cycles were found to arise from: 1) lipids decrease (*ca.* -5%), 2) choline phospholipids increase (*ca.* 7%), 3) Ala, glucose and pyruvate increases (*ca.* 2-7%) and acetone decrease (*ca.* -16%). These results are in broad agreement with previous studies of 1 FT cycle[3], which reported unspecified alterations in lipids, Ala, glucose and lactate. A more recent study[19] on serum noted statistically relevant (p value < 0.05) changes upon 5 and 10 FT cycles (decreases in choline resonance at 3.20 ppm, glycerol, methanol, ethanol, proline, unassigned peak at 1.91 ppm), none of which having been noted in the conditions of this

study. Furthermore, the present study has shown that most variations take place at the fourth FT cycles and thereafter, so that no more than 3 FT cycles are recommendable.

3.4 Evaluation of possible confounding factors: subject non-fasting and long-term plasma storage at -80°C

A cohort of healthy pregnant women in their 2nd trimester of pregnancy, partially matched for age, BMI and gestational age (Table 1), was considered in order to study the effects of -80°C storage for up to 2.5 years. As such samples were collected in non-fasting conditions, a possible confounder superimposed on the eventual effects of long-term storage, the effects of non-fasting were firstly studied on a group of non-pregnant women (Table 1). The PCA plot shown in Figure 4c indicated that, as expected, each subject responded differently to meal intake (see arrows shown as examples); however, no group separation took place, the overall sample groups remaining largely overlapped. Hence, the particular non-fasting conditions characterising sample collection for the pregnant women in this study (2 hours after ingestion of uncontrolled) are not expected to have a strong effect on the overall characteristics of the sample group. Regarding long-term storage at -80°C, multivariate analysis of the spectra for 6-12 months and 14-19 months groups (not shown) detected no significant changes, minor changes being noted when comparing the 14-19 months to 20-30 months groups. This was expressed by a weak non-predictive PLS model (Q^2 0.28) (Figure 4b), which upon inspection of the spectra translated into a small (*ca.* 2%) increase in cholesterol ($p = 0.023$) and slight variations in N-acetyl glycoproteins and creatine (found to be largely determined by two outliers with larger BMI and gestational age, respectively), in the 20-30 months storage period. The above results expressed the low degree of change induced on plasma composition, as viewed by NMR, by the non-fasting conditions used in this work and

by storage up to 30 months at -80°C, after which samples may safely be studied (although interpreting with care eventual changes in cholesterol, after 20 months). Previous NMR results have shown changes in amino acids resonances and the disappearance of citrate resonances in rat plasma stored at -80ºC for 9 months[14], whereas -20°C storage of bovine plasma for between 2-15 years revealed changes in lipids and several low M_W metabolites (glycerol, 3-hydroxybutyrate, amino acids)[16]. To our knowledge, no long-term storage study of human plasma had been carried out before by NMR, a very recent report existing on the subject, using ultra high performance liquid chromatography coupled to mass spectrometry (UPLC-MS)[11], and reporting no significant changes.

4. CONCLUSIONS

In this work, we have shown that EDTA and heparin tubes seem equally suitable for plasma collection for NMR analysis, as long as 1) the level of interfering peaks (in higher number in EDTA tubes) is kept low compared to sample peaks of interest and 2) the loss of sample resonances overlapping with EDTA peaks (and removed from the dataset) is affordable. The latter is particularly important for compounds not giving rise to other resonances (citrate, free choline and dimethylamine) and choline-containing compounds PC, lyso-PC and SM, which are not easily studied in other spectral regions such as the aliphatic or glyceryl regions.

Room temperature stability studies revealed significant changes in the lipidic components of plasma, after *ca.* 2.5 hours. Such changes indicated choline formation (increased up to 20%) through enzymatic cleavage of phospholipids, along with LDL/VLDL conversion into HDL (increase of *ca.* 6 % in HDL/(LDL+VLDL) ratio), possibly also in connection with the increase noted in PC, LPC and SM levels. In

addition to these compounds, no other significant changes in plasma composition were observed at room temperature (up to 21 hours). In relation to short-term (up to 1 month) storage stability, results revealed that plasma samples may be kept at least up to 7 days at -20^{o}C without significant changes occurring. Upon 1 month of storage, increases in proline and glucose and a decrease of a broad resonance at 6.8-7.1 ppm (possibly reflecting protein precipitation) were observed for samples kept at -20^{o}C. For samples stored at -80^{o}C for the same length of time, only the latter variation was observed. In relation to FT cycles (-80^{o}C), the effects of up to 5 cycles were found to be strongly sample dependent, a larger impact noted for a sample richer in lipids. Most variations were seen to take place upon cycle 4 and thereafter, so that no more than 3 FT cycles are recommendable. Changes comprised small decreases in lipids and acetone and increases in choline compounds, Ala, glucose and pyruvate. The longer-term stability of human plasma, at -80^{o}C (up to 2.5 years), was studied on a large cohort (n=49) of pregnant women, for whom plasma collection was performed under non-fasting conditions. In order to investigate the possible confounding effect of non-fasting, this factor was singled out first and studied on a smaller group of subjects (n=16), proving not to affect the overall characteristics of the group of samples, as viewed by multivariate analysis. Subsequently, the effects of long-term -80^{o}C storage were found almost negligible up to 30 months, a small increase in cholesterol having been noted after 20 months storage.

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† Electronic Supplementary Information (ESI) available: ^{1}H NMR spectra of blank solutions in EDTA and heparin collection tubes; overlaid expansions of the ^{1}H NMR spectra of human plasma recorded at room temperature as a function of time; histogram of metabolites varying during plasma storage at-20^{o}C and -80^{o}C for up to 1 month.

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FIGURE CAPTIONS

Figure 1. (a) Superimposed standard ^{1}H NMR spectra of plasma from the same subject, collected with an EDTA tube (black) and a sodium heparin tube (grey). Peak legend: (1) -NC$\underline{H}_2$CO- of $CaEDTA^{2-}$, (2) -NC$\underline{H}_2$CO- of $MgEDTA^{2-}$, (3) -NC$\underline{H}_2CH_2$N- of $CaEDTA^{2-}$, (4) -NCH_2CO- of free EDTA, (5) -NCH_2CH_2N- of $MgEDTA^{2-}$ overlapped with -$N(CH_3)_3$ of choline-containing metabolites, (6) -NCH_2CH_2N- of free EDTA (assignment of EDTA resonances based on reference 12); (b) PCA scores plot of the standard ^{1}H NMR spectra of plasma collected into EDTA tubes (■) and into sodium heparin tubes (□), after exclusion of the regions accommodating EDTA peaks (2.50-2.60 ppm, 2.66-2.72 ppm, 3.04-3.28 ppm and 3.53-3.65 ppm), filled in grey in Figure 1a.

Figure 2. (a) PCA scores plot obtained for the plasma CPMG ^{1}H NMR spectra of subjects 6, 7 and 8, up to 21h at room temperature; (b) Average (of three subjects) CPMG ^{1}H NMR spectrum, colored for coefficient of variation (CV) as determined for the overall 21h period; (c) Heatmap representing the average CPMG integrals of the varying metabolites from minimum (dark blue) to maximum (dark red) values; metabolites are ordered from those exhibiting decreases (above) to those showing increases (below); in the case of largely overlapped regions, the chemical shift indicated corresponds to the maximum intensity observed.

Figure 3. (a) 1D STOCSY obtained using the–$N(CH_3)_3$ choline peak at 3.194 ppm as root peak (see arrow in inset), with color scale expressing the correlation (r) value; (b) Expansions of the –HC=CH region of the average CPMG (left) and diffusion-edited

(right) ^{1}H NMR spectra recorded as a function of time, at room temperature (only selected times are shown).

Figure 4. (a) PCA scores plot obtained for the CPMG ^{1}H NMR spectra of three plasma samples (subject 1, 2 and 3) stored at -20ºC (filled symbols) or at -80ºC (open symbols) for up to 31 days; (b) PCA scores plot obtained for the standard ^{1}H NMR spectra of three plasma samples (subjects 9, 10 and 11) subjected to consecutive freeze-thaw cycles (numbers indicate number of cycles); (c) PCA scores plot obtained for the standard ^{1}H NMR spectra of plasma samples obtained for the same set of subjects (n=16, Table 1), under fasting (■) and non-fasting (□) conditions; arrows represent changes from fasting to non-fasting for selected subjects. (d) PLS-DA scores plot obtained for standard ^{1}H NMR spectra of plasma samples stored at -80ºC for 14-19 months (◆, n=17) and 20-30 months (◇, n=21).

TABLES

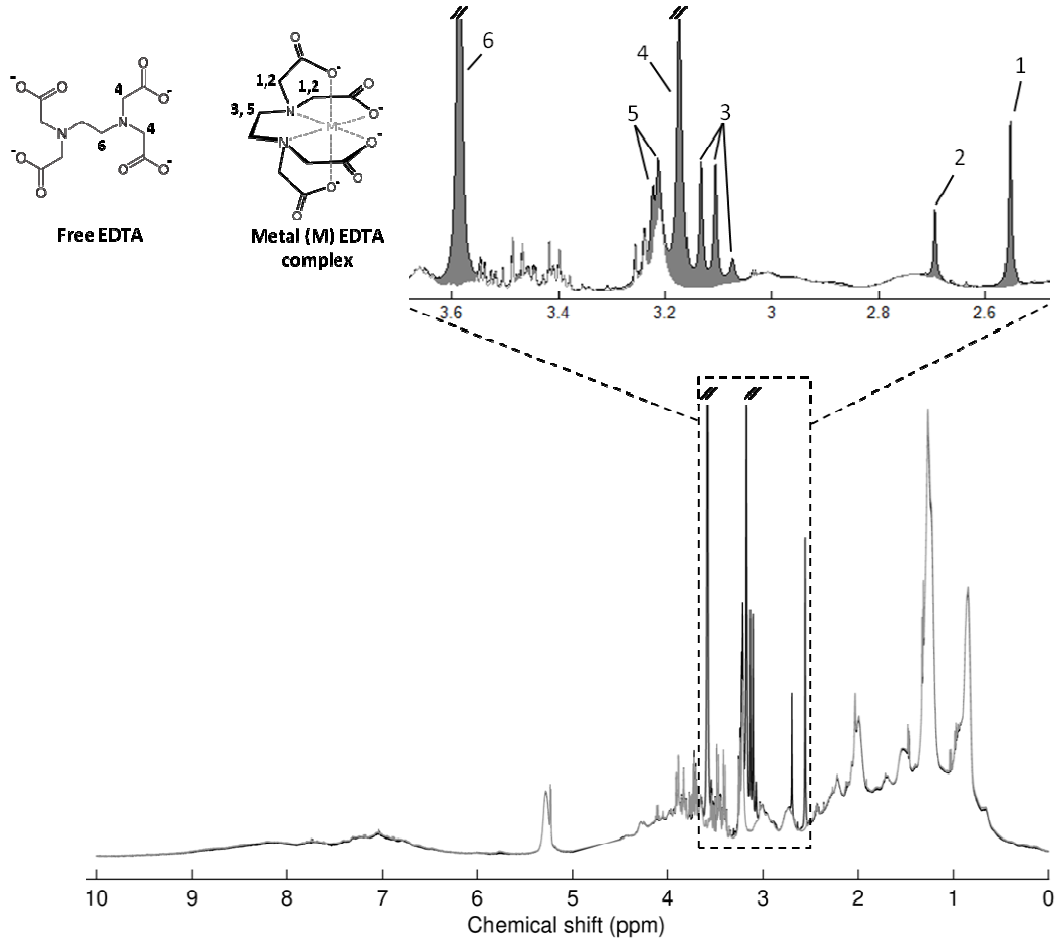
Table 1. Blood plasma sample groups and corresponding identification of studies, number of subjects, gender (all women), age and body mass index (BMI). In columns Age and BMI, numbers in brackets indicate average values, for groups with larger number of subjects. The last column indicates that, for some studies, plasma samples were collected from healthy pregnant women in their second trimester of pregnancy (between 16 and 24 gestational weeks, g.w.).

Study	No. subjects	Gender	Age /years	BMI /Kg.m^{-2}	Observations
Anti-coagulant collection tubes	5	F	23 - 49	19.8 - 34.8	-
Room temperature stability	3	F	29 - 37	21.8 - 32.5	Pregnant (16 g.w.)
Short-term -20^{o}C and -80^{o}C storage stability	3	F	25 - 35	19.8 - 34.8	-
Freeze-thaw cycles	3	F	33 - 36	20.9 - 26.4	Pregnant (17 g.w.)
Fasting and non-fasting	16	F	21 - 36 (27.4)	17.8 - 26.6 (22.0)	-
Long-term -80^{o}C storage					
6-12 months	11	F	25-41 (34.1)	19.8-27.7 (23.6)	Pregnant (16-22 g.w.)
14-19 months	17	F	28-42 (35.7)	20.4-25.6 (23.3)	Pregnant (16-20 g.w.)
20-30 months	21	F	31-42 (36.3)	20.3-33.0 (24.0)	Pregnant (16-24 g.w.)

FIGURES

Figure 1

(a)



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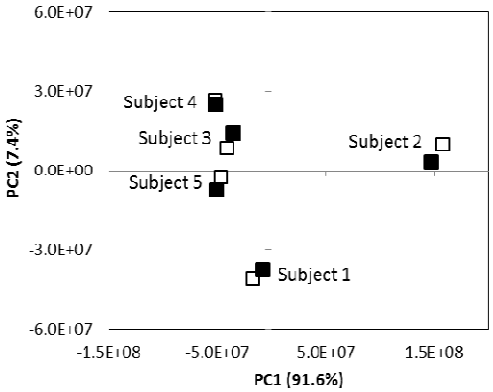


Figure 2

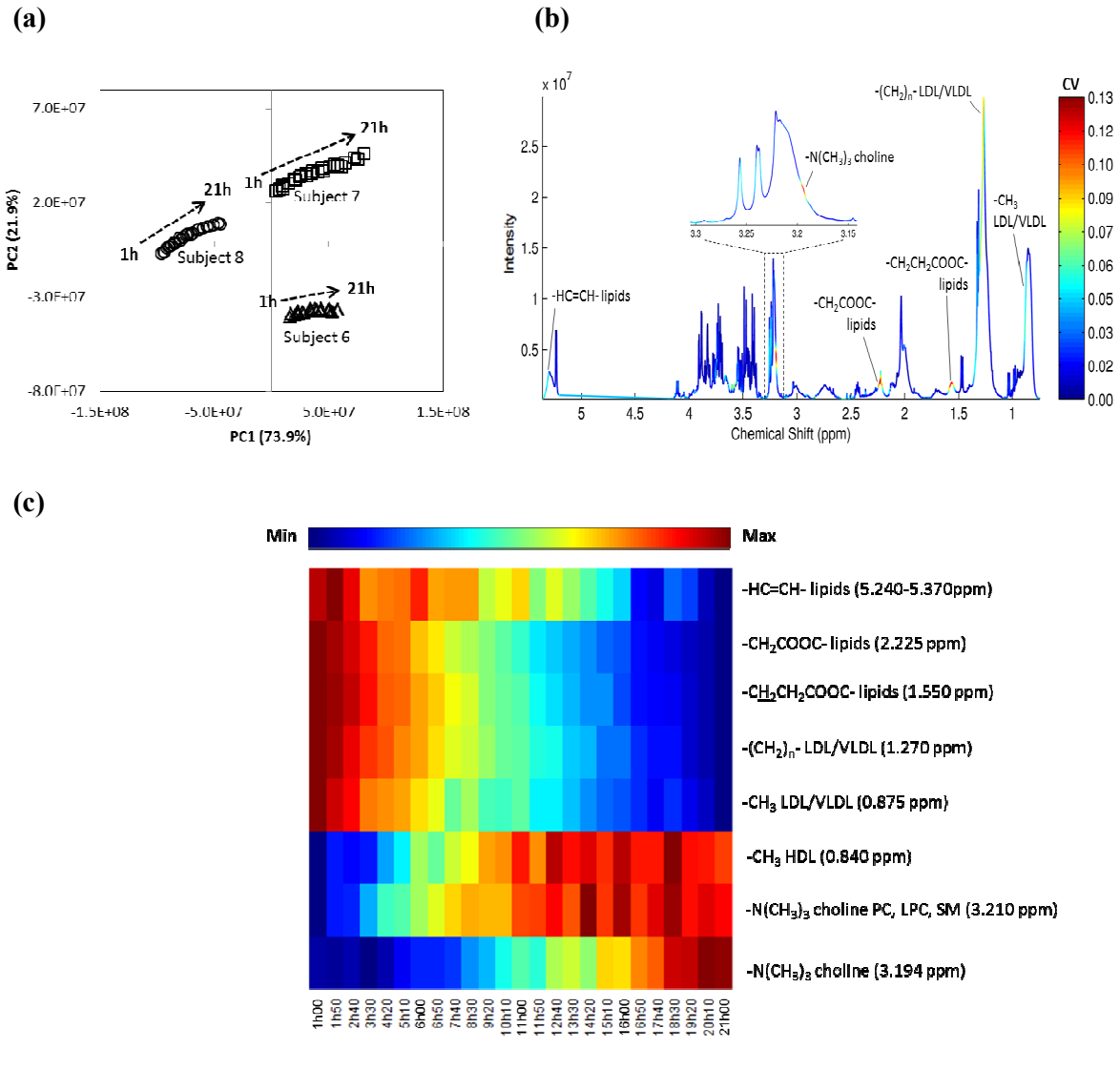
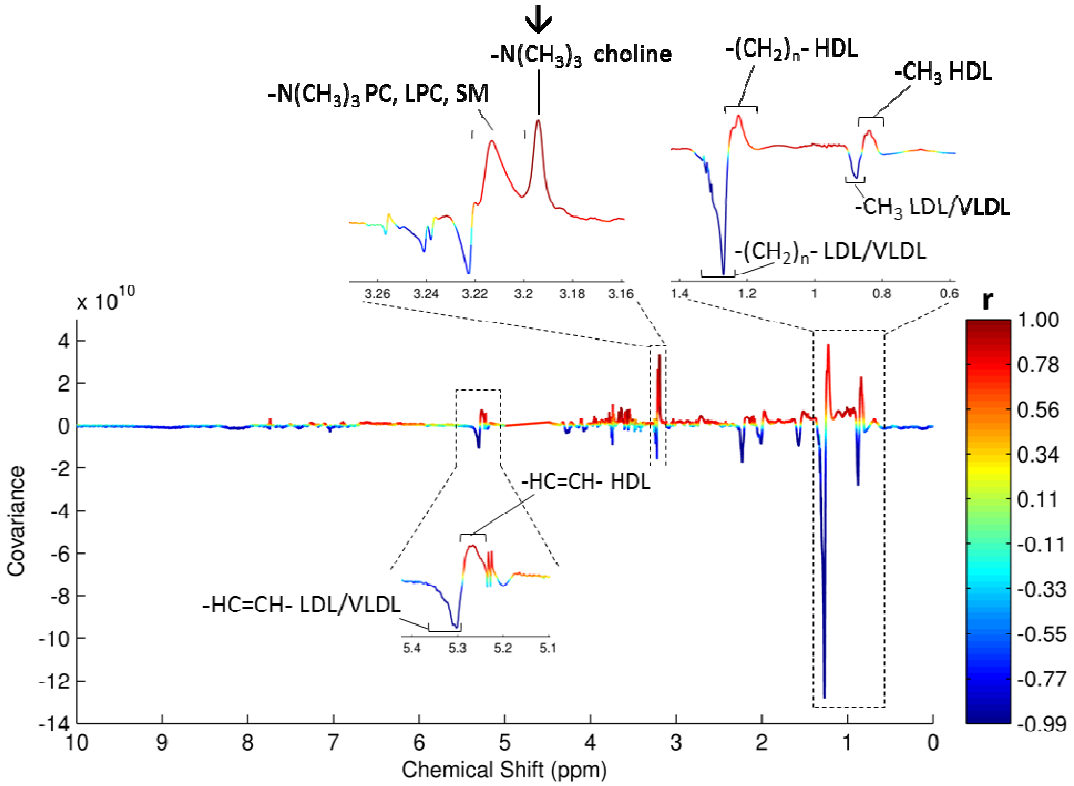


Figure 3

(a)



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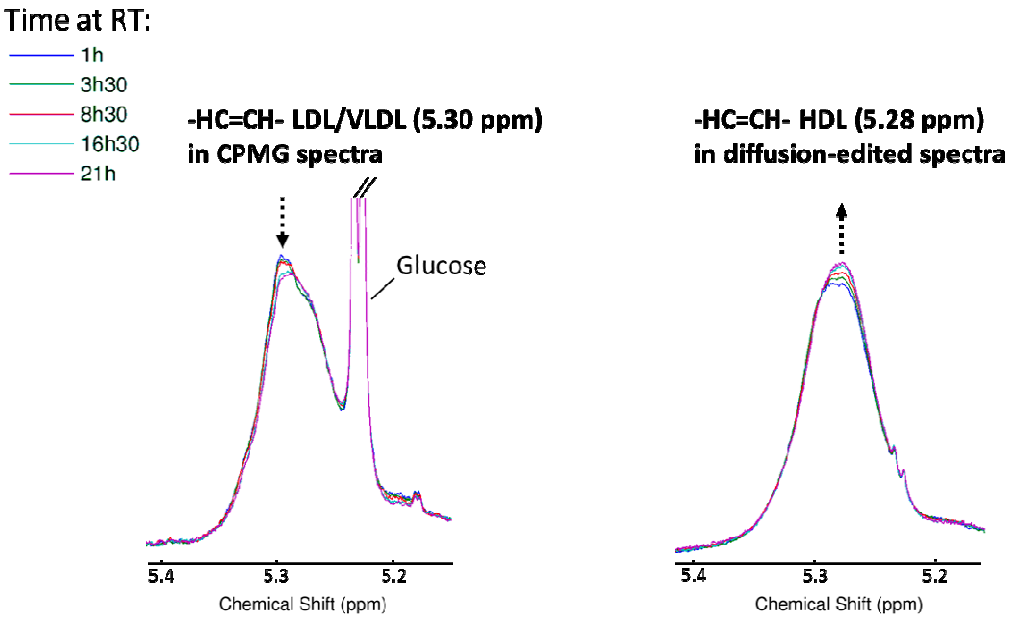


Figure 4

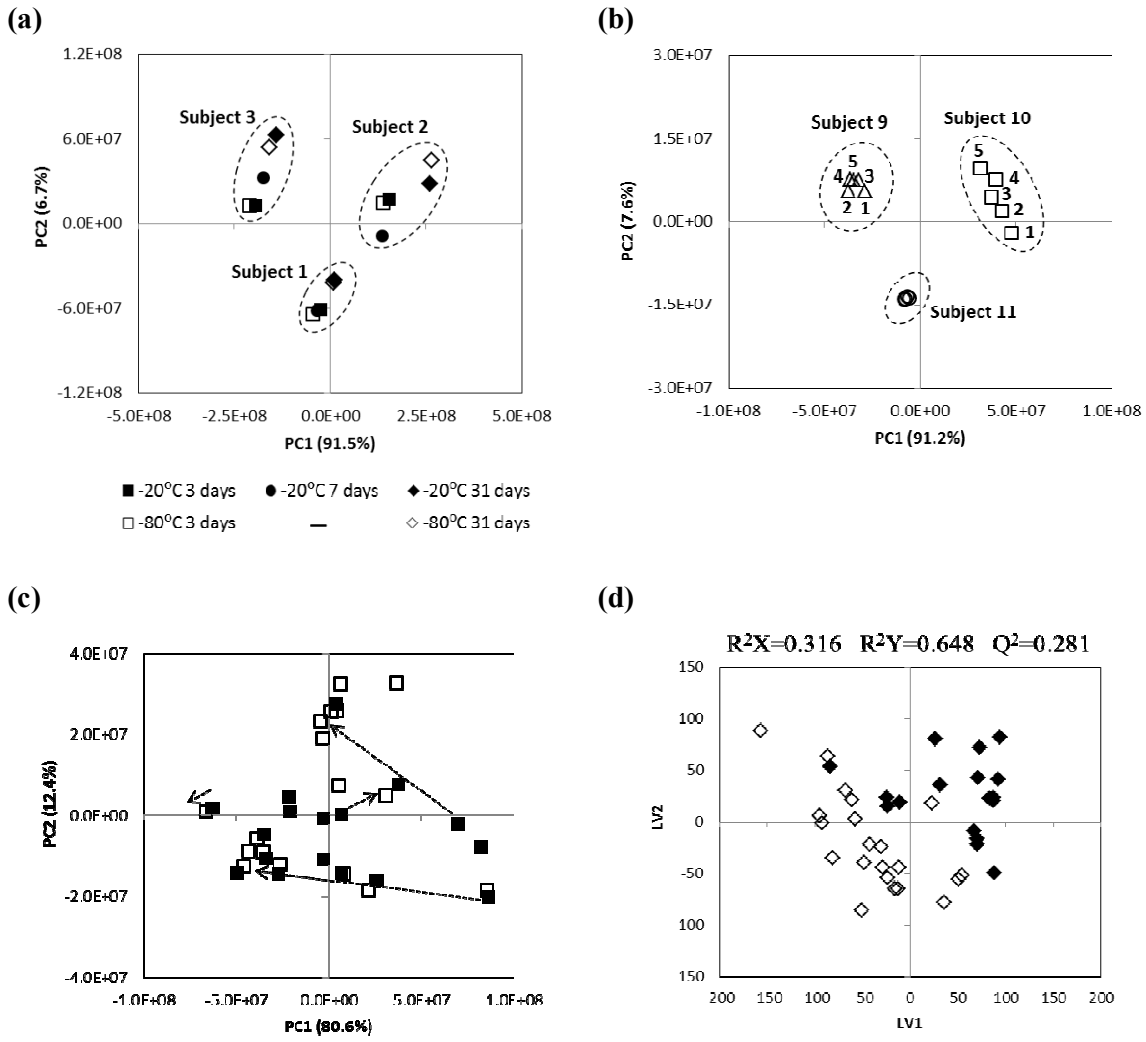
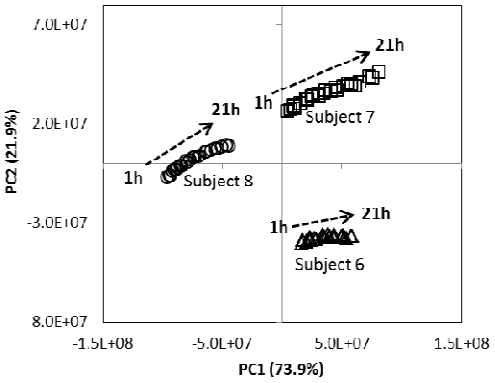


Table of Contents

The stability of human plasma composition was investigated by NMR, considering different collection tubes, time at room temperature (RT), short- and long-term storage conditions and up to 5 consecutive freeze-thaw cycles.

PCA of plasma spectra at RT



Heatmap of signal integrals as a function of time at RT