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A plasma global metabolic profiling approach applied to an exercise study monitoring the effects of glucose, galactose and fructose drinks during post-exercise recovery

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ABSTRACT

A global metabolic profiling methodology based on gas chromatography coupled to time-of-flight mass spectrometry (GC–TOFMS) for human plasma was applied to a human exercise study focused on the effects of beverages containing glucose, galactose, or fructose taken after exercise and throughout a recovery period of 6 h and 45 min. One group of 10 well trained male cyclists performed 3 experimental sessions on separate days (randomized, single center). After performing a standardized depletion protocol on a bicycle, subjects consumed one of three different beverages: maltodextrin (MD) + glucose (2:1 ratio), MD + galactose (2:1), and MD + fructose (2:1), consumed at an average of ~1.25 g of carbohydrate (CHO) ingested per minute. Blood was taken straight after exercise and every 45 min within the recovery phase. With the resulting blood plasma, insulin, free fatty acid (FFA) profile, glucose, and GC–TOFMS global metabolic profiling measurements were performed. The resulting profiling data was able to match the results obtained from the other clinical measurements with the addition of being able to follow many different metabolites throughout the recovery period. The data quality was assessed, with all the labelled internal standards yielding values of <15% CV for all samples (n = 335), apart from the labelled sucrose which gave a value of 15.19%. Differences between recovery treatments including the appearance of galactonic acid from the galactose based beverage were also highlighted.

1. Introduction

The study of physiological interactions between exercise metabolism and intake of macronutrients has been an important area for many researchers over the years, and still continues to be of great interest right up until the present day [1–5]. Macronutrients (carbohydrates, proteins, and fats) and their relation to energy metabolism are also of great commercial interest, not only in relation to commercially available sports "performance enhancing" products, but also in relation to "personalized nutrition" and dietary management for disorders such as type 2 diabetes [6]. There have been a large number of studies (pre-clinical and clinical) designed in an attempt to understand specific mechanisms of action and the effects (e.g. recovery and performance) that such macronutrients have on the body after ingestion [7–9]. The data generated from almost all of these studies generally consists of measurements of one or just a few metabolites either in urine, blood (serum or plasma) and sometimes muscle tissue biopsy samples. Such targeted approaches are generally devised via pre-selected hypotheses that the final results will either statistically support or not. Although this is a sound approach that has been applied for many years, the processes involved at the metabolic level in the recovery period after exercise are very complex, and without a valid methodology in addition to screen all the metabolites, many molecular processes involved after exercise and ingestion of macronutrients will not be observed.

Within nutrition, the "biomics" technologies (e.g. genomics, trancriptomics, proteomics, and metabolomics) are slowly beginning to establish themselves, although up until now mainly only in the form of genomics [10–12]. Nutritional studies focusing

Abbreviations: GC–TOFMS, gas chromatography time-of-flight mass spectrometry; MD, maltodextrin; CHO, carbohydrate; FFA, free fatty acid; IS, internal standard; TMS, trimethylsilyl; Wmax, maximal workload; VO2max, maximal oxygen uptake; MR, magnetic resonance; H-MCR, hierarchical multi-curve resolution; PCA, principal component analysis; AUC, area under the curve; SE, standard error; ANOVA, analysis of variance; OPLS-DA, orthogonal projections to latent structures discriminant analysis; CH, Switzerland.

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on macronutrient intake have also mainly utilised only genomic technology, exploring the effects that macronutrients have on gene expression [13]. The amount of information that has been generated by genomics-based technologies over recent years has provided new insights and tools for human nutrition and food science [12]. However, the latest addition to the omics approaches to emerge; metabolomics/metabonomics [14,15] is concerned with measuring levels of all the metabolites present in bio-fluids (the metabolome) [16,17]. The metabolome can be regarded as the ultimate response of an organism to genetic alterations, disease, diet, and environmental influences. The state of a cell and therefore the phenotypic response exhibited by an organism is directly connected to levels of metabolites. Although other omics approaches have produced large quantities of data, without knowing what is present at the metabolic level, we will never have the full biological picture. Only one group has currently applied a metabolomics approach to a nutritional study investigating the intake of macronutrients on physical exercise [18,19], here a gas chromatography coupled to time-of-flight mass spectrometry (GC–TOFMS) approach was used to assess the metabolic changes observed in human serum within a relatively short recovery period (90 min) after exercise and intake of a post-exercise recovery beverage. This hypothesis free approach allowed multiple levels of metabolites present in the serum to be followed over the recovery time, allowing a 'holistic' metabolic view without being restricted to measurements made on just one or a few metabolites.

Within the field of metabolomics two techniques remain dominant: ^{1}H nuclear magnetic resonance (NMR) spectroscopy; and mass spectrometry (MS) coupled to either liquid or gas chromatography. Both these techniques are regarded as complementary; with NMR displaying superior reproducibility and high sample throughput ability, but MS approaches possess much higher levels of sensitivity, allowing detection of metabolites that appear below the detection limits for NMR based methods. GC–MS methods are widely used for metabolomic based studies [20,21], providing efficient and reproducible analysis. However, a robust and efficient workflow, from sample preparation through to data processing/handling and modelling must be in place to allow a reliable and accurate result [18].

Within the present study, a GC–TOFMS based metabolomics approach was applied to a human exercise study focusing on the effects of three different carbohydrate based recovery beverages, containing either: maltodextrin (MD) + glucose (2:1 ratio), MD + galactose (2:1), and MD + fructose (2:1), consumed after a session of ergometer cycling. Human plasma samples were analyzed for 10 subjects over a recovery period of 6 h and 45 min. The strategy presented here allowed a much greater insight into the metabolic patterns observed in the recovery period, and also could be validated against some of the other clinical measurements that were made. As well as observing the expected changes of energy related metabolites over time, other important metabolite levels were also "captured" within the metabolomic screening approach presented here.

2. Material and methods

2.1. Products and chemicals

Products were obtained from the following suppliers: maltodextrin (Glucidex, Roquette Frères, Lestrem, France), crystalline fructose (Fructose S, Galam Ltd., Basel, Switzerland), D-(+)-glucose (Roferose, Roquette Frères, Lestrem, France) and were mixed at the Nestlé Research Center (Lausanne, CH). HPLC grade methanol and water were obtained from Biosolve (Westford, MA). The stable isotope internal standard compounds (IS): [$^{2}H_4$]-Succinic acid was purchased from Cambridge Isotope Laboratories (Andover, MA), [$^{2}H_6$]-Salicylic acid, [$^{13}C_4$]-Palmitic acid, and [$^{13}C_{12}$]-D-Sucrose were purchased from Isotec, Sigma–Aldrich (St. Louis, MO). Stock solutions of the IS compounds were prepared at a concentration of 500 ng/μL in methanol and kept at 4 °C. N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) plus 1% trimethylchlorosilane (TMCS) was purchased from Pierce (Socochim, Lausanne, CH), pyridine, alkane standard solution C8–C20, alkane standard solution C21–C40, and methyl stearate were purchased from Fluka (St. Louis, MO). O-methylhydroxylamine hydrochloride was purchased from Aldrich (St. Louis, MO). Heptane and hexane were purchased from Sigma (St. Louis, MO). A 500 mL pooled bag of blood plasma used as quality control (QC) plasma was obtained from the CHUV (University Hospital, Lausanne, CH), aliquoted (300 μL) into micro-centrifuge tubes and stored at −80 °C until required for analysis. Blood samples from the trial were collected into pre-chilled appropriately coated tubes (EDTA tubes for free fatty acid measurements and GC-TOF metabolomic analysis, and heparinized tubes for the measurement of glucose and insulin) and centrifuged (3500 rpm, 10 min, 4 °C); samples were stored at −80 °C until further analysis. The total amount of blood taken per volunteer was approximately 80 mL per trial.

2.2. Subjects, preliminary testing and study design

Ten healthy, male endurance trained volunteers were enrolled in the study (mean ± standard deviation: height 184 ± 8 cm, weight 74 ± 8 kg, age 29 ± 5 years, BMI 22 ± 2 [kg/m^2], VO2 max 64 ± 3 mL/kg/min, Wmax 373 ± 42 W).

Prior to the study, volunteers completed a medical examination and anthropometric measurements (body mass, height). Subsequently, volunteers performed a standard graded exercise test to volitional exhaustion at the Department for Cardiovascular Prevention and Rehabilitation (KARE) at the Inselspital (Bern, CH) to determine maximal workload (Wmax) and maximal oxygen uptake (VO2max). The test was performed on an electrically braked cycle ergometer (Excalibur Sport, Lode, Groningen, NL) and started at a work rate of 100 W for 5 min after which the work rate was increased every 2 min by 30 W until the volunteer could no longer maintain the required pedal frequency (>60 rpm). Oxygen consumption and carbon dioxide production were monitored continuously throughout the test using an online automated gas analysis system.

The study was carried out as a double blind, randomized, single center clinical trial. Each volunteer completed three experimental sessions on separate days, consuming one of three separate isoenergetic and iso-caloric beverages for each experimental session: (maltodextrin (MD) + glucose (2:1 ratio), MD + galactose (2:1), and MD + fructose (2:1). The drinks were prepared at Nestlé Research Center in powder form and conditioned in individual sachets. The sachets contained 45 g of carbohydrate powder. They were labelled (A, B or C) and provided to the magnetic resonance (MR) center in a blinded fashion to both the MR staff and the volunteers. The maltodextrin to monosaccharide ratio in the final blends was checked by NUK Quality Assurance Laboratory to be 2:1 for each of the three blends. On the day of each experimental trial, 10 sachets (450 g) were dissolved in 3 L of water plus some drops of lemon juice according to the subject's taste.

The volunteers arrived at the laboratory in the morning after an overnight fast and underwent a cycling exercise protocol that started with a 10 min warm-up at 50% maximal power output. Thereafter, the volunteers were asked to cycle for 2 min periods alternating between 90% and 50% Wmax, until they were no longer able to complete 2 min at 90% Wmax. At this point, the high exer-

cise block was reduced to 80% Wmax, and the alternating process continued until this intensity could no longer be maintained, after which the high exercise block was reduced to 70% Wmax. When the volunteers were no longer able to switch between 70 and 50% in 2 min intervals, they were motivated to maintain levels of workload that were still tolerated (typically 50%) until complete exhaustion. Water was provided ad libitum during the exercise protocol. After finishing the protocol, participants were allowed to take a brief shower and asked to return to the laboratory within 30 min after cessation of the exercise protocol. While supine, a Teflon catheter (Venflon 20 G) was inserted into an antecubital vein in the arm in order to allow for repeated blood sampling during the experimental protocol. Next, the volunteer underwent a basal MR-measurement, followed by the extraction of a resting blood sample (7 mL). Volunteers were then asked to consume the first bolus of test drink (t = 0 min, 600 mL, 2 sachets) and received subsequent drinks (1 or 2 boluses) approximately every 45 min. The average rates of carbohydrate and fluid intake during the 6 h and 45 min recovery period were 1.15 g/min and 460 mL/h respectively (total amount of carbohydrate = 450 g).

Plasma was analyzed at specified times (t = 0, 45, 85, 140, 185, 220, 275, 320, 355, and 405 min) for glucose (enzymatic colorimetric, Wako Bioproducts, Richmond, VA) using an autoanalyzer (XPAND, Dade Behring, Inc.). Plasma insulin was determined by ELISA (IBL, Immuno-biological Laboratories, D-Hamburg). Total plasma free fatty acid content (FFA profile, enzymatic colorimetric, Wako Bioproducts, Richmond, VA) using an autoanalyzer (XPAND, Dade Behring Inc., Eschborn, D). The exercise sampling/schematic protocol is outlined in supplementary material (Fig. 1).

2.3. GC–TOFMS analysis

A 90% methanol extraction mix solution was initially prepared [22] containing [$^2H_4$]-Succinic acid (1 ng/μL), [$^2H_6$]-Salicylic acid (0.5 ng/μL), [$^{13}C_4$]-Palmitic acid (4 ng/μL), and [$^{13}C_{12}$]-D-Sucrose (4 ng/μL). Frozen plasma samples were thawed on ice, centrifuged (3500 rpm, 4 °C, 10 min) and aliquoted (100 μL) into maximum recovery glass vials. The extraction mix solution (900 μL) was added to each plasma, after which the vials were sealed and the samples were vigorously extracted (Vortex Genie 2, highest setting, 30 min, 4 °C). The samples were then centrifuged (3500 rpm, 4 °C, 10 min), and supernatant (200 μL) was taken and transferred into GC–TOFMS analysis vials (with space saver fitting); samples were then evaporated to dryness in a speed-vac (Thermo-Fisher Scientific AG, Wohlen). To the dried samples 30 mg/mL of O-methoxylamine hydrochloride in pyridine was added (30 μL, 2 min vortex), samples were then placed in an oven (70 °C, 60 min) and left for a further 16 h at room temperature. MSTFA 1% TMCS (30 μL) was added to each vial, followed by a short vortex (2 min), and left for 60 min at room temperature. Finally, 30 ng/μL solution of methyl stearate in heptane was added to each vial (30 μL, 2 min vortex), and the samples were now ready for analysis. 1 μL of the derivatized sample extracts was injected in the splitless mode by an Agilent 7683 series autosampler (Agilent, Atlanta, GA) into an Agilent 6980 GC equipped with an inactive phase pre-column (1 m × 0.25 mm) and a DB-5MS (30 m × 0.25 mm × 0.25 μm) stationary phase column (J&W Scientific, Folsom, CA). The injector temperature was set at 250 °C, helium, in constant flow mode (1.4 mL/min) was used as the carrier gas through the column. The column temperature was initially kept at 70 °C for 2 min, then increased at a rate of 15 °C/min until 320 °C where this temperature was held for an additional 11 min. The column effluent was introduced into the ion source of a Pegasus III TOFMS instrument (Leco Corp, St. Joseph, MI). The transfer line temperature was set to 300 °C, and the ion source temperature to 250 °C. Ions were generated using electron ionisation mode (−70 eV), the mass range monitored was m/z 50–500, with an acquisition rate of 25 spectra/s. The samples were prepared in 5 "sample batches", i.e. the whole sample set was prepared and run in 5 separate preparation batches, prepared and injected at one time (n = 335 in total), the batches were created so that they contained a mix of samples from all volunteers, treatments and time points.

2.4. Analysis of GC–TOFMS data

ChromaTOF version 3.25 software (Leco Corp), was used to automatically detect and extract peak areas of the IS and other specific compounds. Non-processed GC–TOFMS raw data files were exported to NetCDF format and read into MATLAB software 7.0.1 (Mathworks, Natick, MA), where all data pre-treatment procedures (baseline correction, chromatogram alignment, and hierarchical multi-curve resolution (H-MCR) via time windows) was performed [23]. Resolved mass spectral profiles from the H-MCR data treatment were compared with spectra from the NIST library version 2.0; and an in-house mass spectral library all using NIST software version 2.0, the identified metabolites, matched from both spectral profile and retention index values are listed in supplementary material (Table 1). The resolved mass spectral profiles were normalized by the injection standard (methyl stearate), correcting for any instrumental drift factors. Multivariate statistical analysis was performed using Simca-P+ version 12.0 (Umetrics AB, Umeå, Sweden). An outline of the GC–TOFMS metabolomic approach is shown in supplementary material (Fig. 2).

3. Results and discussion

3.1. GC–TOFMS data quality

It is essential when performing any metabolomic application/study, that each step within the analysis is carefully planned and assessed. Within the current work, data quality could be observed by utilising labelled internal standards, and an unlabelled "injection standard" (methyl stearate). Labelled internal standards have been utilised in previous metabolomic related studies using both GC–MS [24,25] and LC–MS approaches [26,27]. By adding the methyl stearate (injection standard) we also have a reference that is directly related to the performance/sensitivity of the instrument (the labelled standards undergo the full procedure; extraction and derivatization, as the methyl stearate was added at the end of the preparation). The addition of these labelled compounds and the methyl stearate allows the data quality to be assessed by following the labelled standards. The data also underwent a normalization step via the methyl stearate signal, therefore correcting for instrumental drift factors. This procedure was especially critical as samples were prepared in 5 separate batches (Table 1), and therefore correction for instrumental drift factors is essential. Table 1 shows an improvement in % CV for all internal standards after normalization, with 27 of the 32 measurements (for all samples) falling below 15%. The performance of [$^{13}C_4$]-palmitic acid, and [$^{13}C_{12}$]-D-sucrose within first 2 batches were relatively poor, within these batches, this could be attributed to some instrumental drift factors during the batch runs that were greater than any experienced in the rest of the batches. This can be partially highlighted by the non-normalized response, where the % CV was greater than 25% for both the labelled palmitic acid and sucrose. Overall (all samples n = 335, Table 1), the % CV values all fell below 15%, with the exception of sucrose which was 15.19%.

3.2. Metabolomic data validation utilising clinical data

The FFA profile obtained from the clinical data shows the combined measurement of all FFAs present in the plasma, giving a

Table 1
Data quality assessed by utilizing labelled internal standards (ChromaTOF peak integral data) D_4-succinic acid (251 *m/z*), D_4-salicylic acid (271 *m/z*), $^{13}C_4$-palmitic acid and $^{13}C_{12}$-D-sucrose before and after normalization using the injection standard (methyl stearate (298 *m/z*)). The calculated areas are displayed as 'all samples' ($n = 335$), all the quality control samples ('QCs') only ($n = 24$), all the 'blanks' only ($n = 26$), and all the preparation sample batches (batch 1, $n = 61$; batch 2, $n = 65$; batch 3, $n = 67$; batch 4, $n = 63$; batch 5, $n = 29$) (see Section 2).

	D_4-Succinic acid (251 *m/z*)		D_4-Salicylic acid (271 *m/z*)		$^{13}C_4$-Palmitic acid (332 *m/z*)		$^{13}C_{12}$-D-Sucrose (457 *m/z*)	
	Raw Data (ChromaTOF peak area)	Normalized peak area	Raw Data (ChromaTOF peak area)	Normalized peak area	Raw Data (ChromaTOF peak area)	Normalized peak area	Raw Data (ChromaTOF peak area)	Normalized peak area
All samples ($n = 335$)								
St Dev	155824	0.17	79296	0.08	29024	0.04	86612	0.11
MEAN	676698	1.41	311887	0.64	121050	0.25	355260	0.74
% CV	23	12	25	13	24	15	24	15
QCs Only ($n = 24$)								
St Dev	135566	0.16	66259	0.08	26657	0.03	69321	0.09
MEAN	714654	1.46	329677	0.67	136192	0.28	327130	0.67
% CV	19	11	20	11	20	11	21	13
Blanks Only ($n = 26$)								
St Dev	152938	0.20	65694	0.08	19400	0.03	84068	0.11
MEAN	574150	1.50	218337	0.56	88292	0.23	307548	0.80
% CV	27	13	30	15	22	11	27	14
Batch 1 ($n = 61$)								
St Dev	196343	0.22	95013	0.10	36361	0.05	94596	0.13
MEAN	765903	1.56	357373	0.73	118811	0.24	371945	0.76
% CV	26	14	27	14	31	21	25	17
Batch 2 ($n = 65$)								
St Dev	1792919	0.12	87494	0.07	33454	0.05	69503	0.11
MEAN	583413	1.38	270546	0.63	109334	0.26	265883	0.64
% CV	30	9	32	11	31	20	26	17
Batch 3 ($n = 67$)								
St Dev	73074	0.12	36470	0.06	15532	0.02	47657	0.09
MEAN	703779	1.33	331273	0.62	130627	0.25	420313	0.79
% CV	10	9	11	10	12	9	11	11
Batch 4 ($n = 63$)								
St Dev	115905	0.11	57092	0.06	18418	0.02	50423	0.05
MEAN	691321	1.35	321228	0.63	132954	0.26	370794	0.73
% CV	17	8	18	9	14	7	14	7
Batch 5 ($n = 29$)								
St Dev	86432	0.11	45378	0.05	18307	0.02	55557	0.07
MEAN	653636	1.33	307548	0.63	121002	0.25	402553	0.82
% CV	13	8	15	8	15	9	14	9

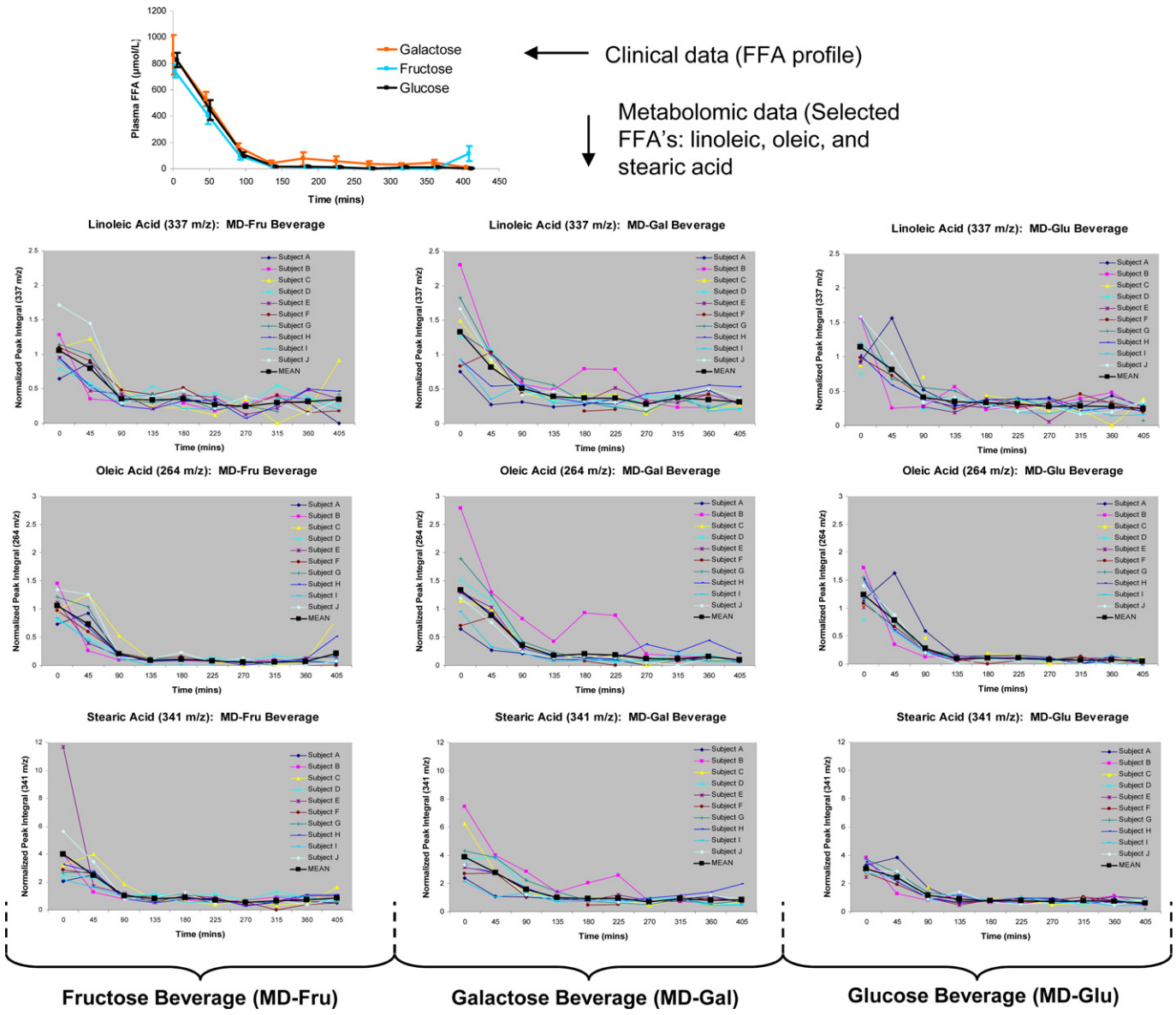

Fig. 1. Comparison of free fatty acid (FFA) clinical data profile and selected (most abundant) FFA measurements from the metabolomic data: linoleic (337 *m*/*z*), oleic (264 *m*/*z*), and stearic acid (341 *m*/*z*) for all 10 subjects A–J. All the metabolomic data is presented as normalized peak integrals, normalized to the injection standard methyl stearate (298 *m*/*z*).

single measurement for each time point per subject, i.e. the overall "FFA pool" was measured and not the individual FFAs. With the metabolomic data, individual FFAs were measured, and could be compared with the obtained clinical data, Fig. 1 shows both the clinical and metabolomic data (only the 3 highest abundant FFAs were used in this comparison: linoleic, oleic, and stearic acid). There were no significant differences observed between treatments in both the clinical and metabolomic FFA results. However, over time the kinetic profiles both show large decreases in FFAs straight after exercise, continuing until the 3rd time point (90 min), after which they stabilized and remained at a constant level until the last time point. The plasma FFA levels during and after exercise have been previously reported [28–30]. It is well known that FFA levels initially fall when commencing exercise, and thereafter gradually increase over time during prolonged exercise. In the recovery period, these levels will gradually fall back to the original levels, here this process is observed to take ~90 min. The reasoning behind this pattern has been attributed to the mechanism of mobilization of FFAs in the blood and therefore available to muscle (FFAs are transported in the blood via albumin and triglycerides). The mobilization processes increase as exercise continues, and eventually more FFAs are mobilized than are taken up and oxidized by muscle. Immediately following cessation of exercise, there is an abrupt rise of FFAs, suggesting that utilization of the FFAs by the muscle comes to an abrupt end, and mobilization factors continue and take more time to eventually establish a new steady state. Within Fig. 1, only measurements in the recovery period were possible here, therefore we observe the slow down of this lipid mobilization process. Within the clinical FFA data, it can be observed that the fructose containing beverage showed a small increase in plasma FFA concentration at the final time point (405 min), and also that the galactose containing beverage displayed slightly higher FFA levels at 180 and 225 min. These increases can also be observed within the metabolomic data, for the fructose containing beverage, the linoleic acid data shows an increase at 405 min for volunteer C, and at the same time point for the oleic acid, volunteers C and H both have increased levels. For the galactose containing beverage, the metabolomic data shows that the increase at 180 and 225 min

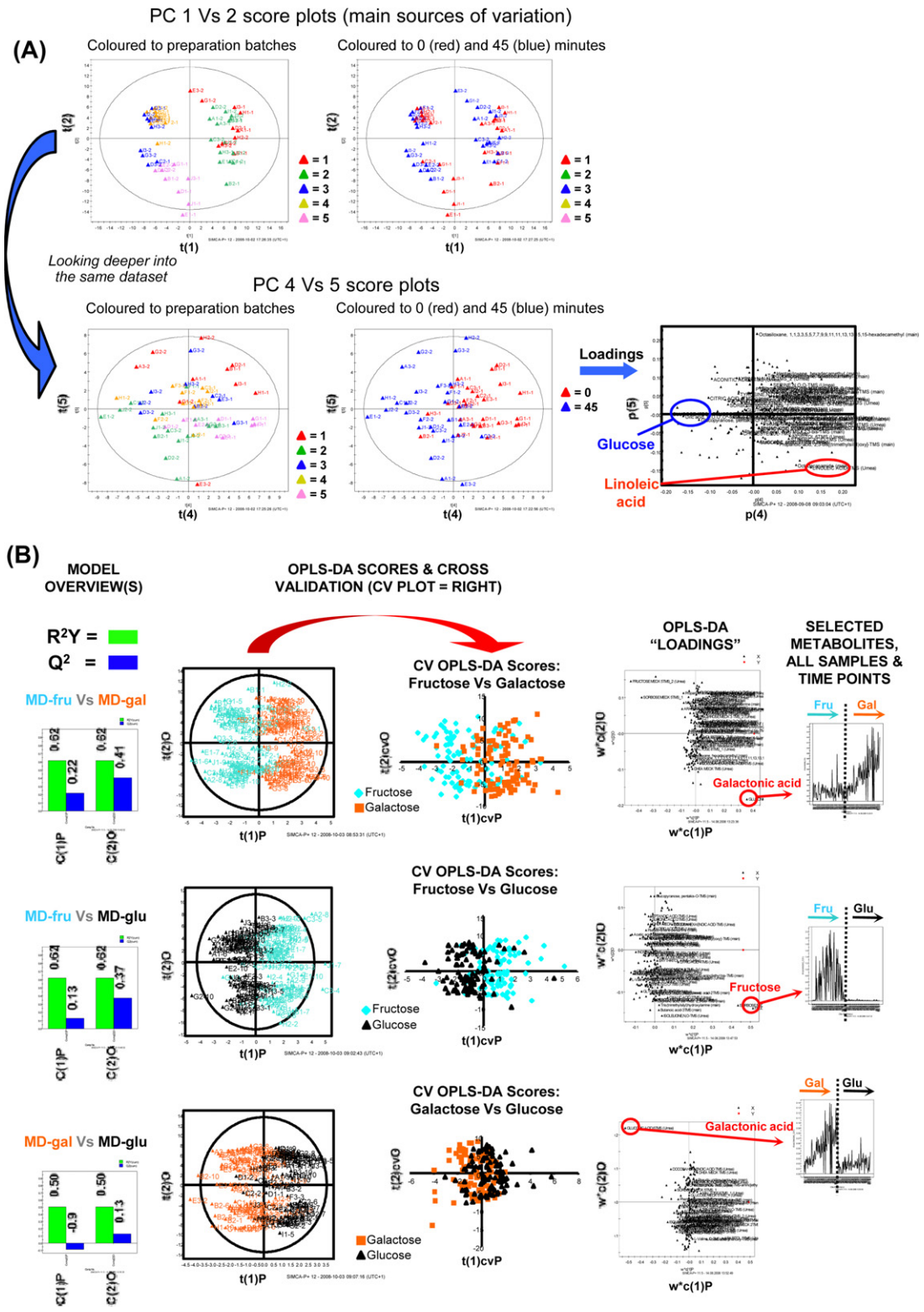

Fig. 2. (A) Unsupervised PCA analysis looking at time dependent changes (0 min vs 45 min, all treatments (i.e. fructose, galactose and glucose treatments) included. (B) Supervised OPLS-DA models looking at inter-treatment variation.

is due to volunteer B, who shows an increase at these two time points for linoleic, oleic and stearic acid. Fig. 1 outlines that the metabolomic data can accurately follow selected metabolites over the recovery period, as is observed with the clinical data, but in more depth, being able to follow individual metabolites and not just a combined profile measurement. The other clinical measurements within the study (glucose and insulin) displayed no inter-treatment statistical differences (supplementary material, Fig. 3). However, it should be noted that observing a further rise in blood glucose levels for the glucose containing beverage would be unlikely due to the already very high levels of glucose present in the blood. These high levels also contribute to instrumental saturation problems within

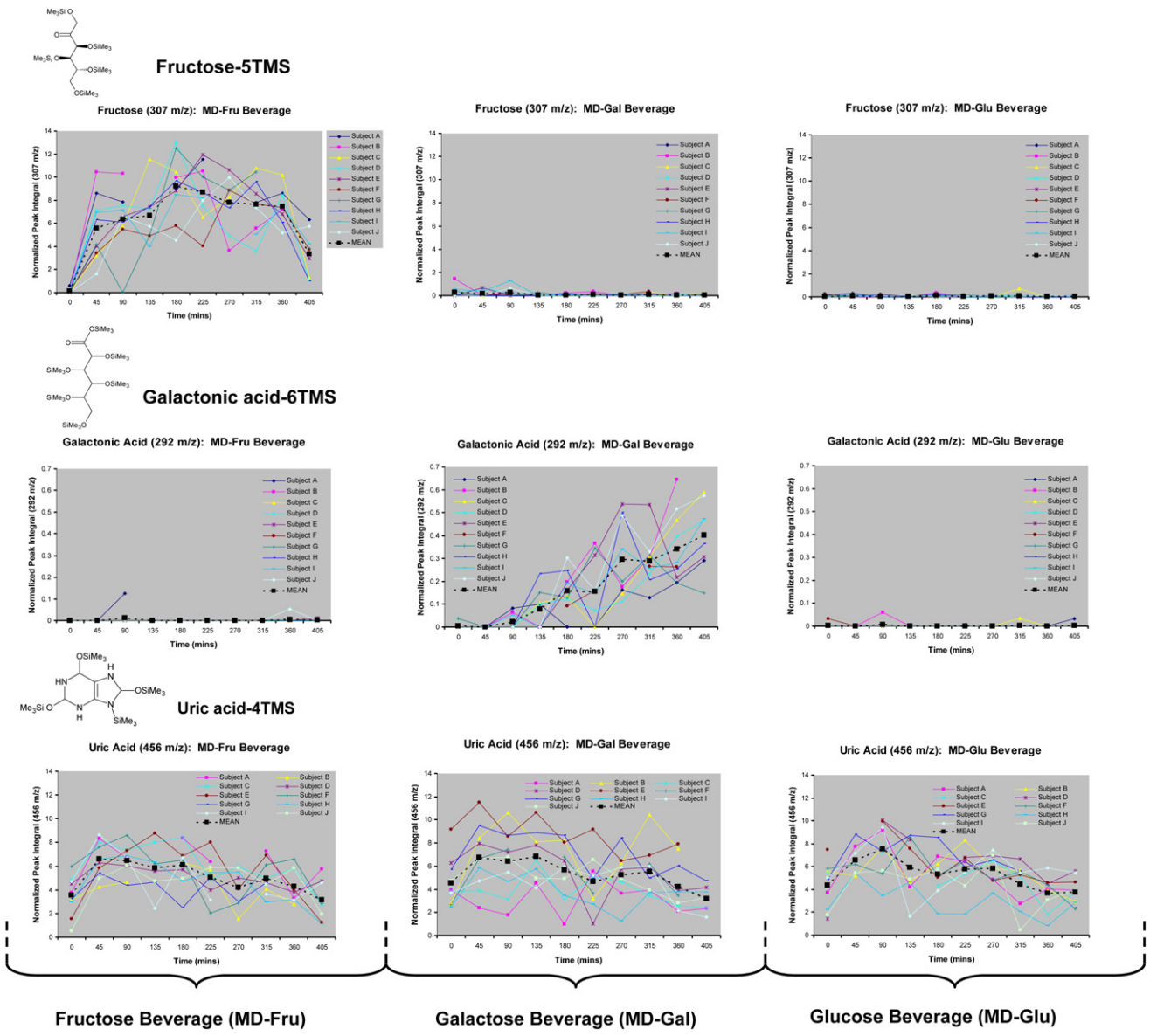

Fig. 3. Normalized peak integrals (normalized using the injection standard methyl stearate (298 *m*/*z*)) from the raw data (fructose (307 *m*/*z*), galactonic acid (292 *m*/*z*), and uric acid (456 *m*/*z*)), looking at the inter treatment comparison (MD-Fru, MD-Gal, and MD-Glu).

the metabolomic analysis, making it even more unlikely to observe differences between treatments for glucose levels.

3.3. *Multivariate statistical analysis*

Overall, 205 chromatographic profiles were extracted with corresponding mass spectra from the metabolomic dataset via the H-MCR procedure (see experimental section). Traditionally, within the field of metabolomics, multivariate statistical analysis is used to model and interpret these large/complex datasets, often in the form of the unsupervised approach of principal components analysis (PCA) and/or the supervised approach of orthogonal projections to latent structures discriminant analysis (OPLS-DA) [31,32]. The present study data was analyzed using both PCA and OPLS-DA approaches (Fig. 2). Within Fig. 2, the PCA (A) shows the changes experienced over the first two time points, with corresponding scores and loadings displaying the changes and the responsible variables, e.g. highlighted in the Fig. 2(A) loadings plot: glucose levels initially increase rapidly and appear much higher at the first time point, as linoleic acid started off high and decreased rapidly over the first 45 min. When applying PCA, the main sources of variation were highlighted by the first few components, with principal component 1 (PC1) explaining 18.8% of the variation, and PC2 = 14.0%. Within Fig. 2, it can be observed that the first 2 component score plots highlight the differences observed between the preparation batches (see experimental section). To observe differences due to time point (within Fig. 2, 0 min vs 45 min), more principal components needed to be explored (4 vs 5), where the scores and corresponding PC 4 vs 5 loadings plot nicely highlight the study samples and discriminating variables (PC4 = 6.1% and PC5 = 4.7%). A problem with applying PCA is that one does not initially know which components will be of interest, particularly when other larger sources of variation can exist. In Fig. 2(B), the supervised OPLS-DA analysis is focused on discriminating between treatments (recovery beverages). The OPLS-DA scores in Fig. 2(B) have also been subjected to cross validation (full cross validation, leave one out method) as the OPLS-DA approach, although a powerful method when attempting to classify treatment groups based on complex datasets like those generated in metabolomics, does tend to over-fit the data, therefore when displaying score plots, this type of valida-

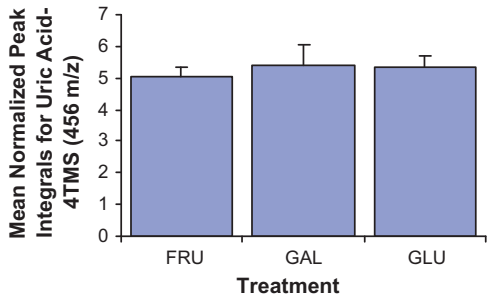

Fig. 4. Mean plasma uric acid levels (0–405 min, $n = 10$ subjects, area under the curve (AUC) ± standard error (SE)) separated into treatment groups (fructose, galactose and glucose). The 456 m/z ion was integrated for uric acid-4TMS (from the GC–TOFMS metabolomic date) using ChromaTOF software.

tion is necessary [33,34]. The OPLS-DA models in Fig. 2 show that many of the metabolites detected do not vary between treatments, therefore the treatments did not cause many observable metabolic differences in the recovery period after exercise. There were "common" changes observed over time (for all 3 treatments), which included amino acids (isoleucine, lysine, valine and tryptophan), which decreased over time, reaching a "low plateau" towards the end of the recovery period. Some metabolites however did change between treatments, rather predictably, but importantly the fructose containing beverage did raise plasma fructose levels in all subjects, something that was not observed in either of the other two beverages. This observation is an important one, although there maybe benefits in administering fructose to the body during and after exercise related to hepatic glycogen levels [35–38], fructose is more lipogenic than glucose, and can cause greater elevations in triglycerides and cholesterol [39].

Dietary fructose has also resulted in increased blood pressure as well as higher levels of uric acid and lactate [39]. Within the metabolomic analysis, uric acid was one of the identified metabolites (Fig. 3), the levels between all treatments (including the fructose containing beverage) were measured, and the result here shows that although the level of fructose in the plasma were high (peaking at around 180 min post exercise), the uric acid levels in all 3 treatments produced similar results (Fig. 4 and Table 2), with no observable differences ($p = 0.776$). This is an important measurement, as high uric acid production via fructose metabolism can be a consequence of either an increase in purine synthesis and/or an increase in nucleoprotein breakdown [40] which can lead to disorders such as hyperglycaemia and gout. However, this result comes after only a single trial of fructose intake. It remains unknown whether consistent oral administration of fructose within a product such as a sports beverage could produce a different result in the long term. The galactose containing beverage also showed high and increasing levels (all the way through the 405 min recovery period) of galactonic acid within the plasma (Fig. 3). Galactonic acid stood out on its own as the only metabolite that showed highly elevated levels in the galactose trial, and also that the levels simply increased over the recovery period (Fig. 3). Galactonic acid has previously been identified as a product of galactose metabolism within humans [41,42] Bergren et al. managed to isolate galactonic acid in the urine of galactosemia patients after being given an oral dose of galactose, the results from this work indicated that there is an oxidative pathway for the metabolism of galactose in human subjects, however they did not measure galactonic acid levels in blood. Observation of high levels of fructose and galactonic acid from galactose metabolism described above in blood via the metabolomic approach does suggest that not all the ingested fructose and galactose was metabolized within the liver. Galactose and glucose are initially absorbed into the liver via a sodium-dependent-glucose-transporter (SGLT1), while fructose is absorbed via a sodium-independent-facilitative-fructose-transporter (GLUT5) [38,43]. Galactose and fructose must then be converted to glucose in the liver before being converted to glycogen, however the present study suggests that the ingested galactose and fructose was possibly too much for the liver to uptake, hence a possible saturation occurred and therefore some of the ingested sugar had a different metabolic fate.

Table 2
Analysis of variance (ANOVA) of uric acid levels from the GC–TOFMS metabolomic data. Determined for each volunteer individually (A–J) inclusive of all 3 treatment groups, and also for each treatment group; fructose (FRU), galactose (GAL) and gluscose (GLU).

MEANS (0–405 min)

Anova: Two-Factor Without Replication

SUMMARY	Count	Sum	Average	Variance
FRU	10	50.39694	5.039694	0.616152
GAL	10	54.11402	5.411402	3.187667
GLU	10	53.26591	5.326591	1.1884
A	3	14.73278	4.910926	2.584749
B	3	16.93285	5.644282	2.612956
C	3	14.92529	4.975096	1.252901
D	3	16.95903	5.653012	0.395745
E	3	20.39725	6.799082	2.906011
F	3	16.27766	5.425887	0.021673
G	3	17.5956	5.865202	2.656215
H	3	11.21065	3.736883	0.715904
I	3	15.02983	5.009944	0.385984
J	3	13.71593	4.571976	0.127045

ANOVA

S. of Var.	SS	df	MS	*F*	*P*-value	*F* crit
Drinks	0.759	2	0.379	0.257	0.776	3.555
Subjects	18.371	9	2.041	1.383	0.266	2.456
Error	26.559	18	1.476			
Total	45.689	29				

The rising levels of galactonic acid from the galactose beverage trial does underline the ability of the methodology described here to be able to detect and follow multiple metabolites over the time course without targeting metabolites. The galactose drink was also run on the GC–TOFMS system used here to check for contamination, or even pre-existing galactonic acid present within the original beverage, however—no galactonic acid was observed, therefore concluding that this molecule was indeed a product of galactose metabolism.

4. Conclusion

The GC–TOFMS based metabolomics approach, including preprocessing via H-MCR and multivariate statistical analysis was successfully applied to studying plasma samples derived from an exercise study focused on the effects of 3 different recovery beverages (MD + fructose, MD + galactose, and MD + glucose). The resulting data was validated by utilizing the additional clinical data, where the more in-depth metabolomic results could follow individual free fatty acids over the time course instead, therefore showing that such an approach is a powerful and accurate method to identify and semi-quantitate multiple metabolites from a variety of different chemical groups within blood plasma. It was also observed that although no differences were seen between treatments within the clinical data, some differences were observed with the GC–TOFMS data. The galactose containing beverage showed increasing levels of galactonic acid throughout the recovery period, and it was also proven that this was not due to contamination of the original beverage, but a product of galactose metabolism determined in the blood.

Also, the fructose containing beverage showed increasing levels of fructose within the blood plasma, although a seemingly obvious observation, it was originally hypothesized that all fructose would be absorbed by the liver and converted to glucose to assist in hepatic glycogen production. The level of fructose was observed to peak at around 180 min into the recovery period, after this a decrease was observed continuing to the final time point, the metabolic fate of this fructose remains unknown. However, the uric acid levels were also compared inter-treatment wise showing no differences between each beverage, therefore clarifying no short term alteration to purine metabolism and possible problems with fructose administration, although the long term affects of administering such a fructose containing beverage remain unanswered.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2010.09.004.

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