research articles Journal of proteome research

^{1}H NMR-Based Metabonomic Investigation of the Effect of Two Different Exercise Sessions on the Metabolic Fingerprint of Human Urine

Alexandros Pechlivanis,[†,||] Sarantos Kostidis,[‡,||] Ploutarchos Saraslanidis,[§] Anatoli Petridou,[§] George Tsalis,[§] Vassilis Mougios,[§,⊥] Helen G. Gika,[†] Emmanuel Mikros,[‡,#] and Georgios A. Theodoridis*[,†]

Department of Chemistry, Aristotle University of Thessaloniki, 54124 Greece, Department of Pharmaceutical Chemistry, School of Pharmacy, National and Kapodistrian University of Athens, 15771 Greece, and Department of Physical Education and Sport Science, Aristotle University of Thessaloniki, 54124 Greece

Received July 2, 2010

Physical exercise modifies animal metabolism profoundly. Until recently, biochemical investigations related to exercise focused on a small number of biomolecules. In the present study, we used a holistic analytical approach to investigate changes in the human urine metabolome elicited by two exercise sessions differing in the duration of the rest interval between repeated efforts. Twelve men performed three sets of two 80 m maximal runs separated by either 10 s or 1 min of rest. Analysis of pre- and postexercise urine samples by ^{1}H NMR spectroscopy and subsequent multivariate statistical analysis revealed alterations in the levels of 22 metabolites. Urine samples were safely classified according to exercise protocol even when applying unsupervised methods of statistical analysis. Separation of pre- from postexercise samples was mainly due to lactate, pyruvate, hypoxanthine, compounds of the Krebs cycle, amino acids, and products of branched-chain amino acid (BCAA) catabolism. Separation of the two rest intervals was mainly due to lactate, pyruvate, alanine, compounds of the Krebs cycle, and 2-oxoacids of BCAA, all of which increased more with the shorter interval. Metabonomics provides a powerful methodology to gain insight in metabolic changes induced by specific training protocols and may thus advance our knowledge of exercise biochemistry.

Keywords: NMR • urine • metabonomics • physical exercise • sprint metabolite profiling

1. Introduction

Physical exercise is a powerful modifier of animal metabolism, as has been shown by numerous studies of the effects of acute and chronic exercise on a plethora of metabolites in a variety of tissues. The overwhelming majority of these studies have performed focused analysis of small numbers of key metabolites, aiming at testing hypotheses regarding specific biochemical pathways.[1,2] In reality, however, various metabolites are involved in several interacting pathways. Capturing a global view of the metabolome and of how the metabolome varies with exercise may help to reveal underlying trends and discover unexpected markers and mechanisms.

Metabonomics represents a holistic, hypothesis-free approach to the study of metabolic responses to various stimuli through powerful data acquisition and advanced data processing techniques that determine a large number of analytes simultaneously.[3] Metabonomics could offer phenotype characterization complementary to genomics or proteomics. Since the metabolic network is downstream of gene expression and, as such, closer to cell activity and function, metabonomics may provide discriminating biomarkers in cases where the knowledge of the genome sequence does not explain a disease progress or reaction to therapeutic intervention.[4,5]

Nuclear Magnetic Resonance (NMR), along with mass spectrometry,[6] offer the major analytical tools in metabolite fingerprinting.[7,8] Although considered a technology of moderate sensitivity, NMR provides important advantages in untargeted metabolite profiling including robustness, high identification power, and superior repeatability and reproducibility.[9,10] Metabonomic data related to physical exercise from either human subjects or animal models are very recent and have mainly focused on blood serum or plasma, examining the effects of strenuous endurance exercise,[11,12] strength-endurance training (rowing),[13] vigorous exercise, and ingestion of a specific diet[14] or postexercise ingestion of different beverages.[15,16] The effects of acute and chronic exercise on the rat liver metabolome[17] and the human urinary metabolome[18] have also been studied. Very recently, a metabolomic approach was applied in the assessment of oxidative stress with strenuous exercise and

* Corresponding author. E-mail: gtheodor@chem.auth.gr.
† Department of Chemistry, Aristotle University of Thessaloniki.
‡ National and Kapodistrian University of Athens.
§ Department of Physical Education and Sport Science, Aristotle University of Thessaloniki.
|| Authors with equal contribution to the research.
⊥ Correspondence for exercise issues: mougios@phed.auth.gr.
Correspondence for NMR analyses: mikros@pharm.uoa.gr.

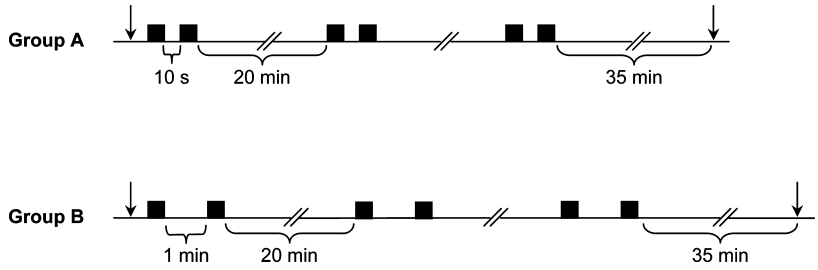

Figure 1. Design of the study. Blocks depict 80 m sprints, and arrows indicate urine sampling. Time is not shown to scale for clarity.

nutritional intervention focusing on the potential beneficial ingestion of *N*-acetylcysteine.[19] In these studies, biomolecules identified as key biomarkers included a number of amino acids, organic acids, and glycerol. Metabonomic approaches have also been applied for the investigation of the effects of exercise on pathological conditions including coronary artery disease,[20] intermittent claudication,[21] and diabetes.[22] The analytical methods applied in these investigations included GC−TOF−MS,[11,13,15,22] CE−MS,[19] LC−MS,[12] and NMR.[14,18,20,21]

In the present paper, we describe a nontargeted, ^{1}H NMR-based metabonomic analysis of urine samples obtained from human subjects before and after two exercise sessions differing in the duration of the rest interval between repeated efforts. This is part of a broader investigation of metabolism during intermittent sprint training, a coaching technique employing repeated short sprints (usually up to 15 s in duration) interspersed with brief rest or low-activity intervals (up to 1 min). By performing sprints of such short duration, athletes simulate the conditions of the race, and their muscles recruit the same energy systems (mainly the ATP−phosphocreatine system and the lactate system) as they do during the real competition. On the other hand, by allowing for only brief recovery intervals that are inadequate for the complete resynthesis of the energy sources used in previous efforts, athletes aim at causing extreme perturbations to the muscle metabolic milieu, thereby eliciting favorable adaptations of the energy systems that power a sprint. Although training with short sprints and with work:recovery ratios between 1:4 and 1:11 has been studied extensively, we could find no data on repeated sprints with very short recovery intervals (work:recovery 1:1), which are used by coaches in modern training practice. We have therefore decided to compare two training protocols containing the same amount of work but with different work:recovery ratios between repeated sprints, i.e., 1:1 vs 1:6. We hypothesized that, because it leaves very little time for the replenishment of energy substrates between efforts, the former protocol elicits greater metabolic disturbances (i.e., differences between pre- and postexercise concentrations of metabolites involved in energy provision) than the latter and may thus provide a stronger stimulus for adaptations. Indeed, the 1:1 protocol caused a larger drop in muscle phosphocreatine and larger increases in muscle glycolytic intermediates than the 1:6 protocol, while being more effective at increasing what coaches refer to as sprint endurance (that is, prolonging the time during which a high running speed can be maintained), as we report elsewhere (Saraslanidis et al., submitted for publication). Here we report on the different effects of the two protocols on the urine metabolome.

2. Experimental Section

2.1. Sample Collection. Twelve young, moderately trained, healthy males provided written informed consent to participate in the study. The study was approved by the institutional ethics committee, and all procedures were in accordance with the Helsinki declaration of 1975, as revised in 1996. The participants were divided into two equivalent groups: group A (age, 21 ± 2; body mass, 69 ± 7 kg; height, 1.79 ± 0.04 m; BMI 22.1 ± 1.9 kg/m^2; all mean ± SD) and group B (age, 20 ± 1; body mass, 71 ± 7 kg; height, 1.79 ± 0.06 m, BMI 22.0 ± 1.5 kg/m^2). These groups were then randomly assigned to either of two exercise sessions. Each session included three sets of two 80 m maximal runs. The two runs in each set were separated by either 10 s (in group A) or 1 min (in group B) of rest, and sets were separated by 20 min of rest in both groups. Urine was collected before and 35 min after completion of each exercise session. Urine samples were divided into aliquots which were stored at −80 °C until analysis. The experimental design is depicted in Figure 1.

To control for the effect of nutrition on substrate utilization during exercise, the participants were given standard dietary plans to be followed during the two days preceding exercise testing. The plans provided 50% of energy from carbohydrate, 35% from fat, and 15% from protein. On the morning before exercise testing, they ate a standardized meal. To control for the effect of hydration status on urine production, apart from the standardized meal (which contained 0.5 L of water), the participants consumed 1 L of tap water between the pre- and postexercise samplings.

2.2. Sample Preparation. An aliquot of each sample was used for metabonomic analysis through ^{1}H NMR spectroscopy. Sample pretreatment was kept as simple as possible. The samples were thawed just before analysis and were centrifuged at 1500*g* for 5 min. An amount of 300 μL of the supernatant was mixed with 300 μL of phosphate buffer, pH 7.4, in D_2O-H_2O 70:30 (v/v) containing 0.684 mmol/L sodium 3-trimethylsilyl-(2,2,3,3-$^2H_4$)-1-propionate (TSP) and 0.2 mg/mL of NaN_3. The mixture was vortexed and transferred into a 5 mm wide NMR tube.

2.3. Creatinine and Lactate Assays. For validation purposes, two major urine metabolites, creatinine and lactate, were determined through spectrophotometry. Creatinine was measured by use of the CREA plus kit from Roche Diagnostics (Mannheim, Germany). Lactate was measured according to former method no. 826-UV from Sigma Diagnostics (St. Louis, MO). Briefly, one volume of urine (in the case of the preexercise samples) or one volume of urine diluted 1:10 with water (in the case of the postexercise samples) was mixed with 30 volumes of a working solution made from 10 mL of 0.6 mol/L glycine buffer, pH 9.2 (Sigma), 20 mL of water, 50 mg of NAD (Applichem, Darmstadt, Germany), and 500 U of lactate dehydrogenase (Sigma). After incubation for 30 min, the NADH formed, being equimolar to the lactate initially present in the sample, was measured at 340 nm by using a molar extinction coefficient of 6.22 L/(mmol cm).

Table 1. Urine Lactate and Creatinine Concentrations, as Determined Spectrophotometrically, in Group A (10 s Interval between Repeated Sprints, $n = 6$) and Group B (1 min Interval, $n = 6$)[a]

	pre-exercise		postexercise				
metabolite	group A	group B	group A	group B	effect of exercise	effect of interval	interaction
lactate (mmol/L)	2.3 ± 1.2	2.3 ± 1.4	134.8 ± 12.7	74.4 ± 32.1	<0.001	<0.001	<0.001
creatinine (mmol/L)	26.2 ± 10.2	27.3 ± 11.9	17.1 ± 3.6	21.6 ± 7.4			
lactate/creatinine	0.10 ± 0.07	0.09 ± 0.05	8.22 ± 2.00	3.56 ± 1.75	<0.001	<0.001	<0.001

[a] Descriptive data are presented as means ± SD. Data in the final three columns are *P* values resulting from two-way ANOVA; only significant ones ($P < 0.05$) are shown.

2.4. ^{1}H NMR Spectroscopy. All NMR experiments were carried out on a Bruker (Karlsruhe, Germany) Avance III 600 MHz spectrometer, employing an inverse detection probe (5 mm) with z-gradients, at 300 K. One-dimensional (1D) ^{1}H NMR spectra were obtained using a standard 1D NOESY pulse sequence (noesypr1d) included in the spectrometer's library, with presaturation during relaxation and mixing time for water suppression. For each spectrum a total of 128 scans was collected in 64-k data points over a spectral width of 7211.5 Hz, using a relaxation delay of 2 s, an acquisition time of 4.54 s, and a mixing time of 0.1 s. An exponential weighting factor corresponding to a line broadening of 0.3 Hz was applied to all acquired free induction decays prior to Fourier transformation and phase correction. All spectra were referenced to the chemical shift of TSP at δ 0.0 ppm and baseline-corrected by applying a polynomial curve fitting ($A + Bx + Cx^2 + Dx^3 + Ex^4$, except for the region of the water signal) using the XWIN-NMR software (Bruker BioSpin GmbH).

Two-dimensional (2D) ^{1}H−^{1}H COSY and TOCSY spectra with water presaturation were acquired for selected samples to assist/confirm the assignment of metabolites' spin systems. The COSY spectra were acquired using a gradient-selected pulse sequence (sine-shaped gradient pulse) in the magnitude mode, with 2048 time domain points and 256 increments, while 8 scans were measured per increment. The spectral width was 12 019 Hz with a 2 s relaxation delay and an acquisition time of 0.08 s in F2. The TOCSY spectra were collected in the phase-sensitive mode using states time proportional phase incrementation (States-TPPI) and the MLEV17[23] pulse sequence for spin lock over 0.07 s. For each 2D spectrum, 2048 time domain points were collected, and 1024 increments were measured with 16 scans per increment. The spectral width was 7211.5 Hz, with a 2 s relaxation delay and an acquisition time of 0.14 s in F2. The data sets were weighted using a sine-bell-square apodization function in t_1 and t_2 prior to Fourier transformation and phase correction. ^{1}H−^{13}C multiplicity edited HSQC (DEPT135-HSQC) 2D NMR spectra were recorded for selected samples, utilizing the sensitivity enhancement in the phase-sensitive, echo-antiecho-TPPI mode with gradient and multiplicity selection and GARP for carbon decoupling during evolution and acquisition. The spectral width was 20 ppm in F2 and 165 ppm in F1, with 1024 time domain points and 512 increments with 64 scans per increment, adiabatic inversion pulses, and a relaxation delay of 1.5 s. The J_{CH} delay was optimized to 145 Hz. 2D *J*-resolved (JRES) spectra were measured with water presaturation, into 8192 data points with a spectral width of 1000 Hz, while *J*-coupling domain spectral width covered 78 Hz with 40 increments and 1 scan per increment. Data were processed with a sine-bell function in t_2 and t_1, tilted by 45°, and symmetrized about the F1 axis.

2.5. Statistical Analysis and Pattern Recognition. All 1D ^{1}H NMR spectra were reduced to a series of descriptors[24] by segmenting the spectral region of δ 0.4−9.6 ppm into regions (buckets) of 12 Hz width (0.02 ppm) using the AMIX software (Analysis of Mixtures version 2.7, Bruker Analytische Messtechnik). The 4.72−4.98 ppm region, containing the residual peak of the suppressed water resonance, was excluded to remove baseline effects of imperfect water suppression. Because the chemical shifts of histidine, 1-methylhistidine, and 3-methylhistidine were susceptible to small pH differences among samples (despite their buffering), the spectral regions which include the shifting resonances of these metabolites (8.17−7.87, 7.15−7.01, and 3.77−3.71) were excluded from data reduction. Histidine, 1-methylhistidine, and 3-methylhistidine signals, at 8.02−7.88, 7.81−7.72, and 8.17−7.95 ppm, respectively, were separately integrated and considered in the univariate statistical analysis. The region of the urea's broad signal (6.05−5.51) was also excluded. The signal intensity of each of the remaining 387 spectral regions was integrated, and data were normalized in two ways, that is, to the sum of intensities and to the creatinine methyl resonance intensity at δ 3.05 ppm, as explained in the Results section. Although normalization of untargeted metabonomics data to creatinine may be hindered by technical and biological difficulties, we observed very stable ^{1}H NMR signals for the molecule.

To identify the variables with the highest influence on the discrimination between pre- and postexercise and between the 10 s and 1 min rest intervals, two-way analysis of variance (ANOVA) on the aggregate signals of each identified compound was performed using the SPSS software (version 15.0, SPSS, Chicago, IL). The level of statistical significance was set at $\alpha = 0.05$.

Spectral data were submitted to pattern recognition analyses with multivariate statistical analysis tools. Principal component analysis (PCA), partial least-squares discriminant analysis (PLS-DA), and orthogonal PLS-DA (OPLS-DA) were performed in SIMCA P11 (Umetrics, Umea, Sweden). PCA provides an unbiased tool to study the data; both univariate and pareto scaling modes were tested. Pareto scaling was finally chosen, as it is more appropriate for analytical spectroscopy data.[25] The supervised methods (PLS-DA, OPLS-DA) were employed mainly to study the contribution of the variables in group separation and to find differentiating biomolecules.

3. Results

3.1. Urine Creatinine and Lactate Concentrations. Targeted assays (section 2.3) showed that there were highly significant main effects of exercise and rest interval, as well as a highly significant interaction of the two variables on lactate concentration (all $P < 0.001$). In Table 1 the urine lactate and creatinine concentrations determined are presented. This statistical outcome is explained by the fact that lactate increased with exercise in both groups, but the increase in group A was 1.8

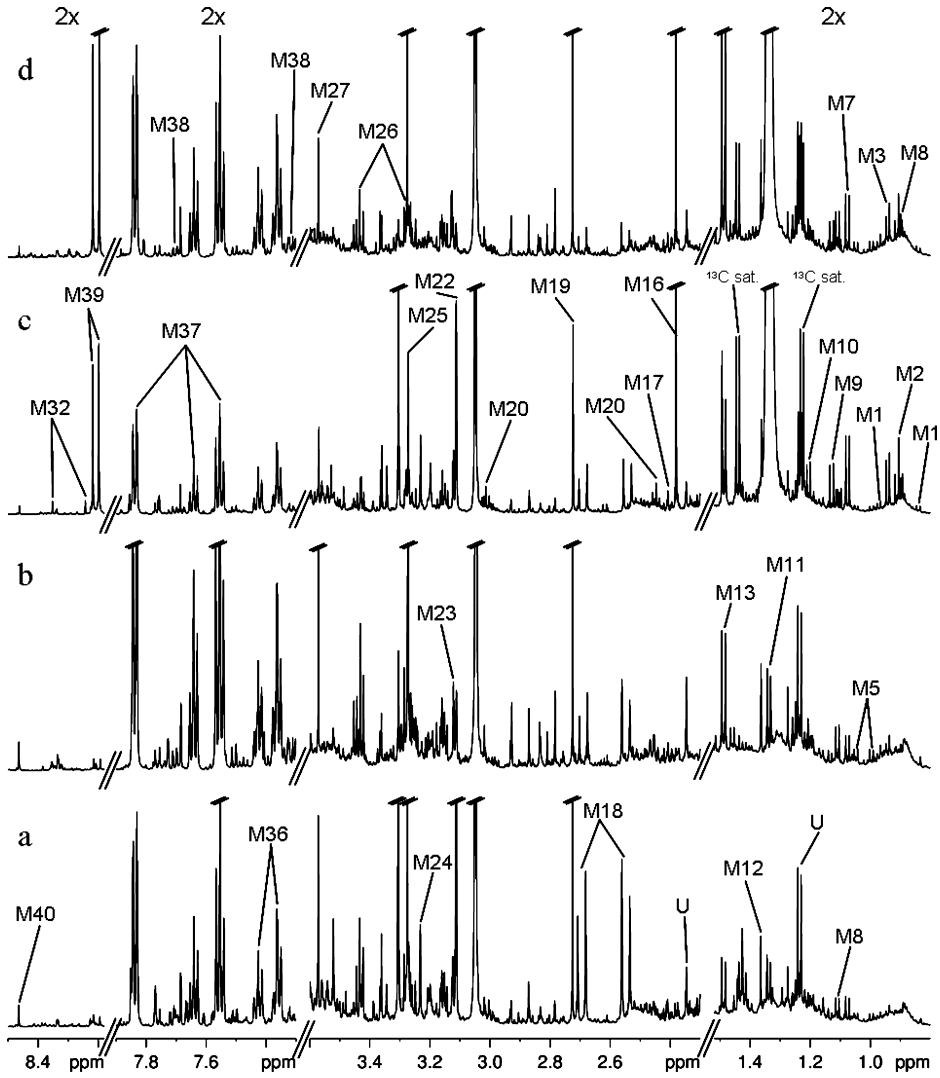

Figure 2. Representative 600 MHz ^{1}H NMR spectra of urine samples. Only spectral regions containing the most significant metabolite resonances are shown. a, pre-exercise group A; b, pre-exercise group B; c, postexercise group A; d, postexercise group B. Key of metabolites is as referred to in Table 2.

times the increase in group B. This difference between groups was even higher (2.3-fold) when the lactate concentration was normalized to the creatinine concentration (a widely used practice in clinical chemistry to account for differences in renal function) resulting in equally significant main effects and interaction. The lactate-to-creatinine ratio was highly correlated to the ^{1}H NMR signal intensity of lactate normalized to creatinine (Spearman's ρ 0.94, $P < 0.001$, Supporting Information Figure S1), thus providing strong evidence for the validity of the quantitative ^{1}H NMR data.

3.2. ^{1}H NMR Spectral Analysis. Urine ^{1}H NMR spectra are highly complex, dominated by numerous signals of low-molecular mass metabolites as depicted in Figure 2 and Supporting Information Figure S2, where characteristic regions and total spectra, respectively, of pre- and postexercise urine samples from both groups are presented. Resonances assignment was carried out according to the literature,[26,27] an in-house database, and spiking experiments with reference compounds (inosine, myoinositol, propionate, hydantoin, adenosine, and hypoxanthine, data not shown), and it was confirmed by 2D COSY, TOCSY, JRES, and DEPT135-HSQC of selected samples. In total, 43 metabolites were identified and subsequently assigned, as listed in Table 2 and Supporting Information Table S1.

3.3. Data Treatment, Normalization, and Multivariate Analysis. Initially, PCA was applied on data normalized to the sum of signal intensities in an attempt to decipher whether an unsupervised method of multivariate statistical analysis can discriminate groups. Postexercise samples were clearly separated from pre-exercise samples mainly due to the massive response of lactate (representing almost half of the total signal intensity). To overcome the influence of the extremely intense lactate signal on the normalization process and to avoid suppression of the contribution of other metabolites in the statistical model, integrals were normalized to the creatinine signal at δ 3.05. The resulting data set provided more robust models exhibiting higher predictability, as suggested by the higher R2X(sum) and Q2(sum) values. Hence normalization to creatinine was chosen, and all further statistical analysis is based on this approach. (For a list of the generated statistical models and their component vector values, see Supporting Information Table S2).

Table 2. Human Urine Metabolites Identified by ^{1}H NMR Spectroscopy and Relative Changes in Metabolite Levels after Exercise in Groups A and B[a]

metabolite key	metabolite	chemical shift (ppm)	group A	group B	effect of exercise	effect of interval	interaction
M1	2-Hydroxyisovalerate	(CH_3) 0.84, (CH_3) 0.97, (CH) 2.03, (CH) 3.85	1.39 ± 0.35	1.14 ± 0.15	0.010		
M2	2-Hydroxybutyrate	(CH_3) 0.90, (CH) 4.01, (CH_2) 1.65/1.74	2.65 ± 0.82	1.58 ± 0.22	<0.001	0.011	0.010
M3	2-Oxoisocaproate	(CH_3) 0.94, (CH) 2.10, (CH_2) 2.62	1.74 ± 0.33	1.26 ± 0.12	<0.001		0.012
M4	Leucine	(CH_3) 0.96, (CH_2) 1.70	1.03 ± 0.25	0.93 ± 0.13			
M5	Valine	(CH_3) 0.99, (CH_3) 1.04, (CH) 2.26, (CH) 3.60	1.00 ± 0.19	0.95 ± 0.12			
M6	Isoleucine	(CH_3) 1.01	1.00 ± 0.18	0.94 ± 0.09			
M7	3-Hydroxyisobutyrate	(CH_3) 1.07, (CH) 2.48, (CH_2) 3.68	1.65 ± 0.37	1.52 ± 0.24	<0.001		
M8	3-Methyl-2-oxovalerate	(CH_3) 0.89, (CH_3) 1.10, (CH_2) 1.45/1.70, (CH) 2.94	1.86 ± 0.38	1.56 ± 0.22	<0.001	0.013	
M9	2-Oxoisovalerate	(CH_3) 1.12, (CH) 3.02	1.34 ± 0.22	1.14 ± 0.23	0.016		
M10	3-Hydroxybutyrate	(CH_3) 1.20, (CH_2) 2.31/2.41, (CH) 4.16	0.92 ± 0.12	0.89 ± 0.11			
M11	Lactate	(CH_3) 1.33, (CH) 4.12	64.14 ± 16.77	29.96 ± 15.73	<0.001	<0.001	<0.001
M12	2-Hydroxyisobutyrate[b]	(CH_3) 1.36					
M13	Alanine	(CH_3) 1.49, (CH) 3.79	2.22 ± 0.39	1.83 ± 0.33	<0.001		
M14	Acetate	(CH_3) 1.93	1.00 ± 0.14	0.93 ± 0.20			
M15	Acetoacetate	(CH_3) 2.24, (CH_2) 3.44	0.88 ± 0.25	0.75 ± 0.21			
M16	Pyruvate	(CH_3) 2.38	3.81 ± 0.73	2.13 ± 0.58	<0.001	<0.001	<0.001
M17	Succinate	(CH_2) 2.41	1.21 ± 0.17	1.07 ± 0.16			
M18	Citrate	(CH_2) 2.51/2.54/2.66/2.69	0.61 ± 0.16	0.63 ± 0.17	0.005	0.018	
M19	Dimethylamine	(CH_3) 2.71	1.05 ± 0.07	0.98 ± 0.11			
M20	2-Oxoglutarate	(CH_2) 2.45/3.01	1.27 ± 0.13	1.15 ± 0.19	0.003	0.002	
M21	Creatinine	(CH_3) 3.05, (CH_2) 4.06					
M22	*cis*-Aconitate[b]	(CH_2) 3.11, (CH) 5.69					
M23	Malonate[b]	(CH_2) 3.12					
M24	Carnitine	(CH_3) 3.21, (CH_2) 2.43, (CH_2) 3.43	1.00 ± 0.28	0.85 ± 0.12			
M25	Trimethylamine *N*-oxide	(CH_3) 3.27	0.79 ± 0.22	0.81 ± 0.15	0.044	0.029	
M26	Taurine	(CH_2) 3.27, (CH_2) 3.43	0.76 ± 0.17	0.85 ± 0.21			
M27	Glycine	(CH) 3.57	0.77 ± 0.13	0.67 ± 0.13	0.014		
M28	*N*-Methylnicotinamide	(CH_3) 4.48, (CH) 8.21, (CH) 8.90, (CH) 8.97, (CH) 9.28	0.71 ± 0.17	0.69 ± 0.16			
M29	Glucuronate[b]	(CH) 4.65/5.26, (CH) 3.29/3.58, (CH) 3.52/3.73, (CH) 3.52, (CH) 4.07/3.73					
M30	Allantoate[b]	(CH) 5.26					
M31	Allantoin	(CH) 5.39	0.78 ± 0.19	0.72 ± 0.18			
M32	Inosine	(CH_2) 3.85/3.92, (CH) 4.28, (CH) 4.44, (CH) 4.78, (CH) 6.10, (CH) 8.24, (CH) 8.35	2.12 ± 0.65	1.81 ± 2.21			
M33	Fumarate	(CH) 6.52	2.66 ± 0.53	1.73 ± 0.33	<0.001		0.026
M34	*trans*-Aconitate	(CH_2) 3.45, (CH) 6.59	1.31 ± 0.25	0.91 ± 0.19			
M35	Tyrosine	(CH_2) 3.02/3.17, (CH) 3.93, (2,6 CH) 6.91, (3,5 CH) 7.17	1.25 ± 0.73	0.80 ± 0.26			
M36	Phenylalanine	(CH_2) 3.17, (CH) 3.98 (2,6 CH) 7.36, (4 CH) 7.39 (3,5 CH) 7.43	0.89 ± 0.18	0.78 ± 0.38			
M37	Hippurate	(CH_2) 3.96, (3,5 CH) 7.56, (4 CH) 7.64, (2,6 CH) 7.84	0.84 ± 0.43	0.73 ± 0.81			
M38	Tryptophan	(CH_2) 3.30/3.42, (CH) 4.02, (CH) 7.19, (CH) 7.27, (CH) 7.33, (CH) 7.54, (CH) 7.71	0.89 ± 0.18	0.76 ± 0.09	0.017	0.031	
M39	Hypoxanthine	(CH) 8.19, (CH) 8.22	7.57 ± 1.30	9.29 ± 4.36	<0.001		
M40	Formate	(CH) 8.46	0.72 ± 0.17	0.68 ± 0.30	0.020		
M41	Histidine	(CH) 7.04−7.14, (CH) 7.88−8.02	0.67 ± 0.18	0.76 ± 0.12	0.014		
M42	1-Methylhistidine	(CH) 6.96−7.05, (CH) 7.72−7.81	0.83 ± 0.09	0.91 ± 0.20			
M43	3-Methylhistidine	(CH) 7.09−7.18, (CH) 7.95−8.17	1.06 ± 0.92	0.52 ± 0.36			

[a] Spin systems of metabolites were assigned with 2D ^{1}H−^{1}H COSY and TOCSY spectra. Descriptive data are presented as means ± SD. Values for each metabolite are signal intensity ratios of post- to pre-exercise after normalization to the creatinine signal intensity. Data in the final three columns are *P* values resulting from two-way ANOVA; only significant ones ($P < 0.05$) are shown. [b] No value is reported due to partial peak overlap.

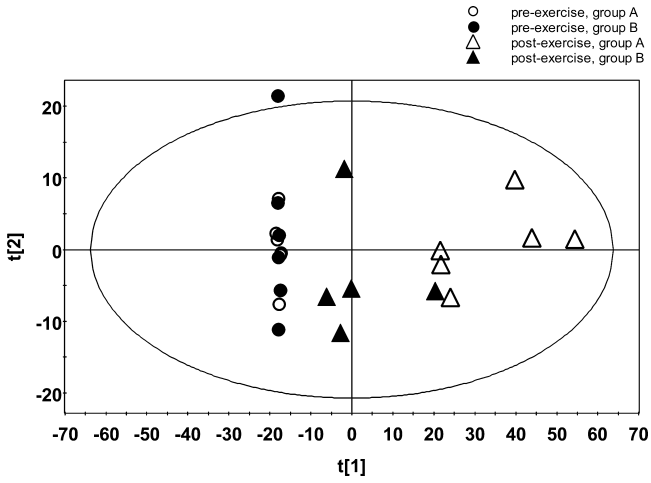

Figure 3. Scores plot after PCA of all urine samples. PC1 accounts for 80.1% of the variation and discriminates pre- from postexercise samples. PC2 accounts for 8.4% of the variation. Hotelling ellipse depicts a 95% confidence interval. One postexercise sample of group B was excluded from the statistical analysis due to an abnormal signal.

3.3.1. Effect of Exercise. When examining all urine samples in one workset, a PCA scores plot (Figure 3) provided clear-cut separation of all pre- from all postexercise samples in PC1 (PC1 accounting for 80.1% of the variation). Furthermore, differentiation of the postexercise samples of each group was evident. Postexercise groups showed higher dispersion in PC1, indicating different responses of the individuals to exercise. In PC2 (8.4%), the variation due to biological variability and genetic or environmental/habitual background is described.

Next, statistical models were built excluding lactate (by deleting the lactate buckets, 4.11−4.15, 1.43−1.45, 1.27−1.39, and 1.23 ppm, from the raw data set before importing into SIMCA P) to reveal the contribution of other discriminating metabolite markers. PCA scores plots still provided separation of pre- from postexercise samples in the first two components (data not shown). In the subsequent statistical analysis of the effect of exercise, only data after exclusion of the lactate signal were used.

To further elucidate the effects of exercise, statistical models were built for each exercise group separately. Figure 4a shows a PCA scores plot for group A where PC1 and PC2 explain 28.3% and 24.4% of the variation, respectively. Separation of pre- from postexercise urine samples is still observed despite the exclusion of lactate and its ^{13}C satellites. These findings were further scrutinized using PLS-DA and OPLS-DA.[29] Both modes provided better group separation (scores plot of PLS-DA data shown in Supporting Information Figure S3) with R2X values higher than 0.56 and R2Y values higher than 0.97, indicating robust statistical models. A permutation test done in SIMCA P showed that the predictive power of the models was satisfactory; however, the small number of observations limited the models' statistical significance (although it should be noted

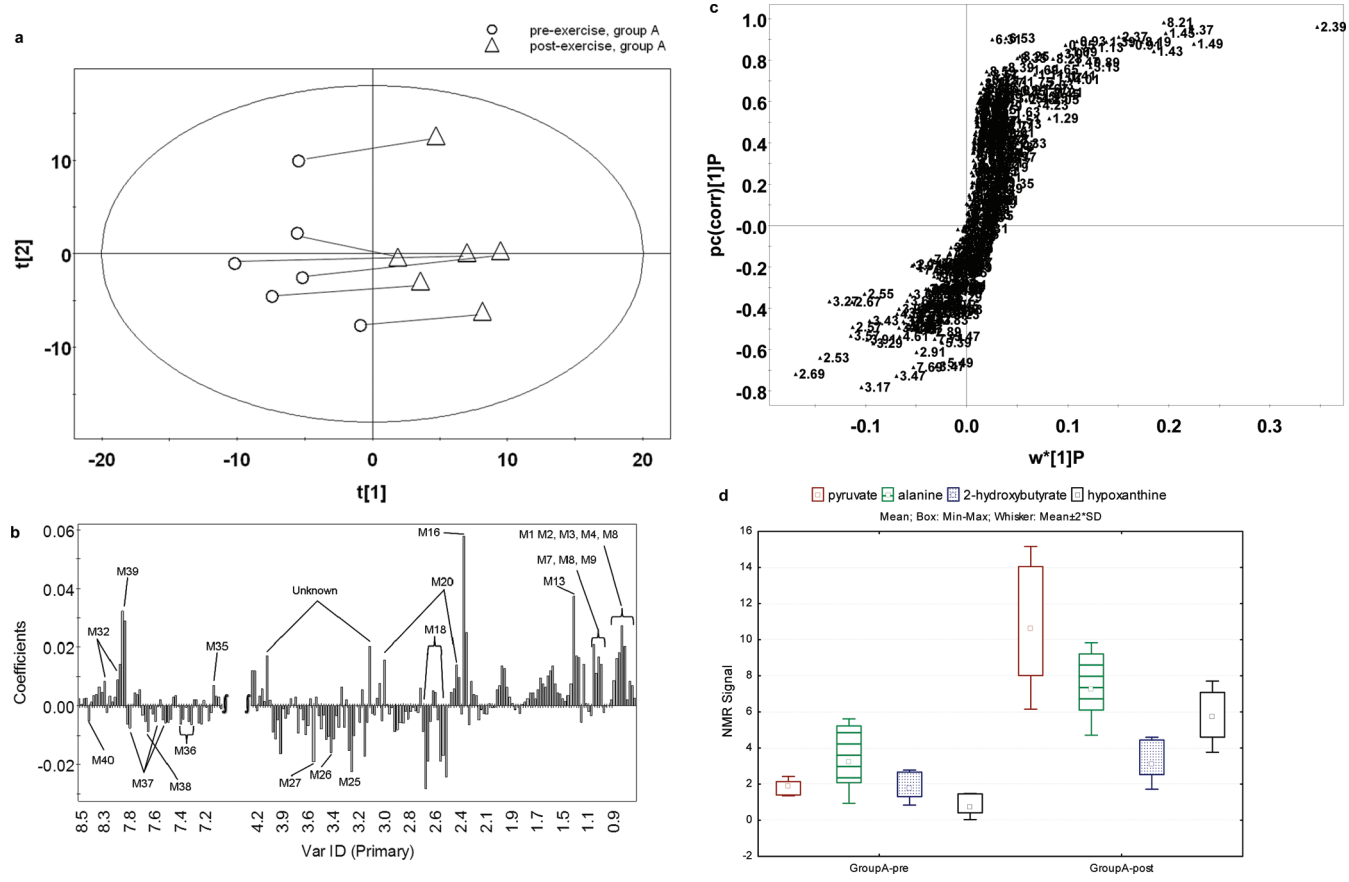

Figure 4. Multivariate statistical analysis of data from the ^1H NMR analysis of urine from group A (10 s interval between maximal runs). (a) PCA scores plot: circles, pre-exercise; triangles, postexercise. PC1: 28.3%. PC2: 24.4%. (b) PLS-DA coefficient plot showing the contribution of the major differentiators. (c) S-plot highlighting differentiators of pre- from postexercise (2.39 corresponds to pyruvate, 2.69 and 2.53 to citrate, etc.; see Table 2). (d) Box plots of NMR signal as ratios normalized to creatinine for selected biomolecules contributing to the differentiation of pre- from postexercise.

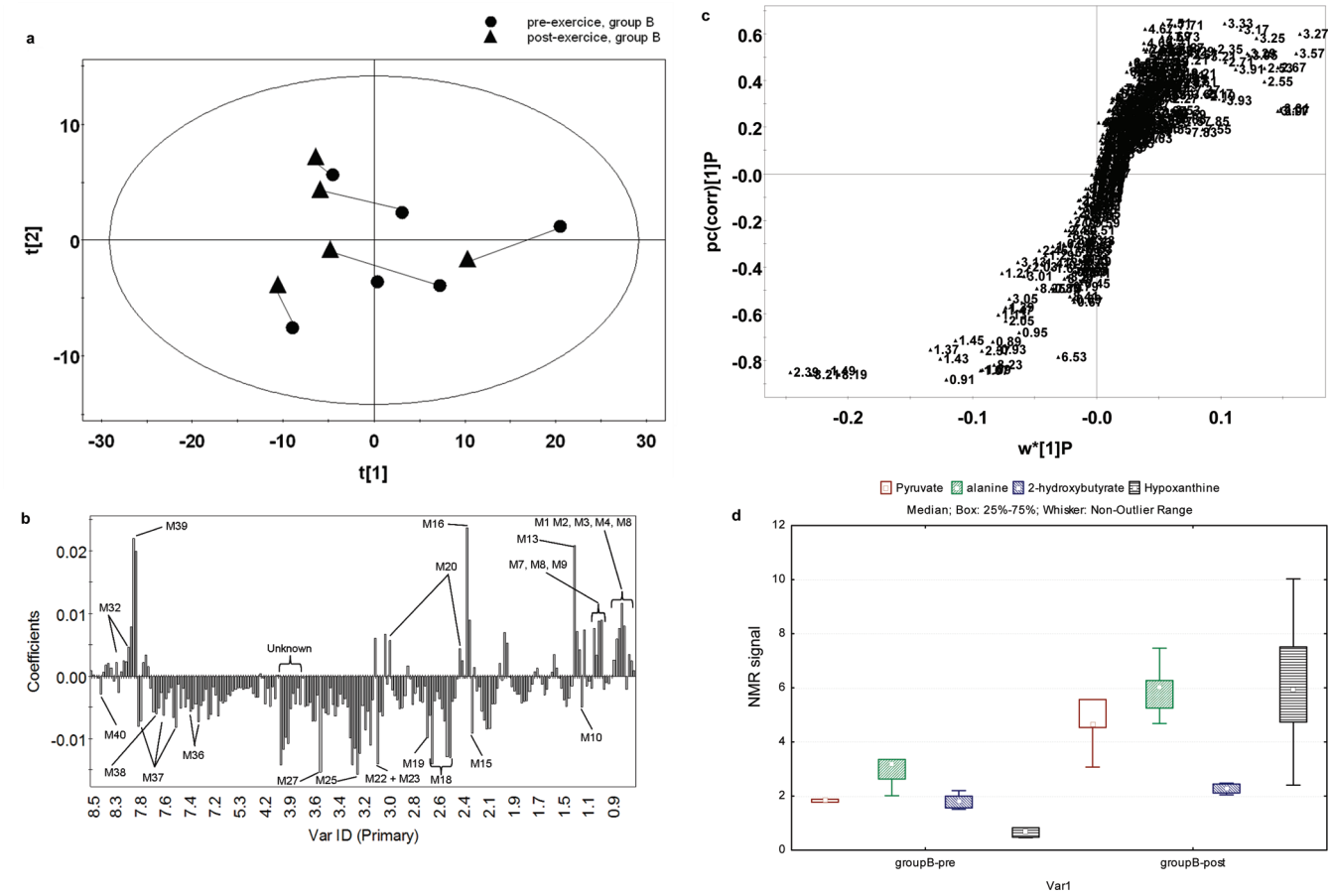

Figure 5. Multivariate statistical analysis of data from the ^{1}H NMR analysis of urine from group B (1 min interval between maximal runs). (a) PCA scores plot: circles, pre-exercise; triangles, postexercise. PC1: 53.1%. PC3: 9.9%. (b) Coefficient plot showing the contribution of the major differentiators. (c) S-plot highlighting differentiators of pre- from postexercise (8.21 and 8.19 correspond to hypoxanthine, 0.91 to 2-hydroxybutyrate, 3.27 to trimethylamine *N*-oxide, etc.; see Table 2). (d) Box plots of NMR signal as ratios normalized to creatinine for selected differentiating metabolites.

that, in the original model including lactate, statistical significance was high). Next, advanced data illustration tools, such as contribution plots, S-plots, and VIP (variable importance in the projection) values, were employed to identify the features contributing to group separation. Figure 4b is a PLS-DA coefficient plot showing the markers with the highest contribution to group separation. The S-plot (generated from the OPLS-DA model) shows variables contributing highly to the differentiation to be located at the ends of the plot (Figure 4c). These findings were cross-checked by examining the raw spectral data. Metabolites that were showing reliable signal differentiation between the groups (as illustrated by PCA trends plots or by using box plots) were considered as potential markers. As an example, Figure 4d shows box plots from NMR raw data (peak integrals) for selected differentiating biomolecules that exhibited an increase after exercise, while Figure S4 (Supporting Information) shows trends plots for tryptophan, fumarate, glycine, pyruvate, and 2-hydroxybutyrate.

Similar thorough examination was applied to the data of group B. In the PCA (scores plot PC1 vs PC3, Figure 5a), pre- and postexercise samples were not clearly separated (PC1 accounted for 53.1% of the variation, PC3 for 9.9%). PLS-DA or OPLS-DA scores plots maximized group differentiation (scores plot of PLS-DA data shown in Supporting Information Figure S5) with R2X values higher than 0.62 and R2Y values higher than 0.92, indicating robust statistical models. In addition, permutation tests showed good predictive ability. Figure 5b shows the coefficient plot along with assignment of the major differentiators. Figure 5c shows an S-plot generated from an OPLS-DA model. Major differentiators were hypoxanthine and pyruvate (up-regulated). Box plots of the NMR signal for a selected number of metabolites are given in Figure 5d.

From the above multivariate analysis, it was found that 22 out of the 43 assigned metabolites changed significantly in both groups after exercise. Thirteen increased, including five products of BCAA catabolism (2-hydroxyisovalerate, 2-oxoisocaproate, 3-hydroxyisobutyrate, 3-methyl-2-oxovalerate, and 2-oxoisovalerate), 2-hydroxybutyrate, lactate, alanine, pyruvate, 2-oxoglutarate, inosine, fumarate, and hypoxanthine. Nine of these (2-oxoisocaproate, 3-methyl-2-oxovalerate, 2-oxoisovalerate, 2-hydroxybutyrate, lactate, alanine, pyruvate, 2-oxoglutarate, and fumarate) increased to a higher magnitude in group A than in group B, whereas one (hypoxanthine) showed higher increase in group B than in group A. This can explain the clearer separation of pre- from postexercise samples in PCA for group A. Nine metabolites decreased, including citrate, trimethylamine *N*-oxide, taurine, glycine, allantoin, phenylalanine, hippurate, tryptophan, and formate. Of these, glycine showed a higher decrease in group B.

3.3.2. Effect of Rest Interval. To study the hypothesis that a 10 s rest interval between maximal sprints elicits greater metabolic disturbances than a 1 min interval, two new statisti-

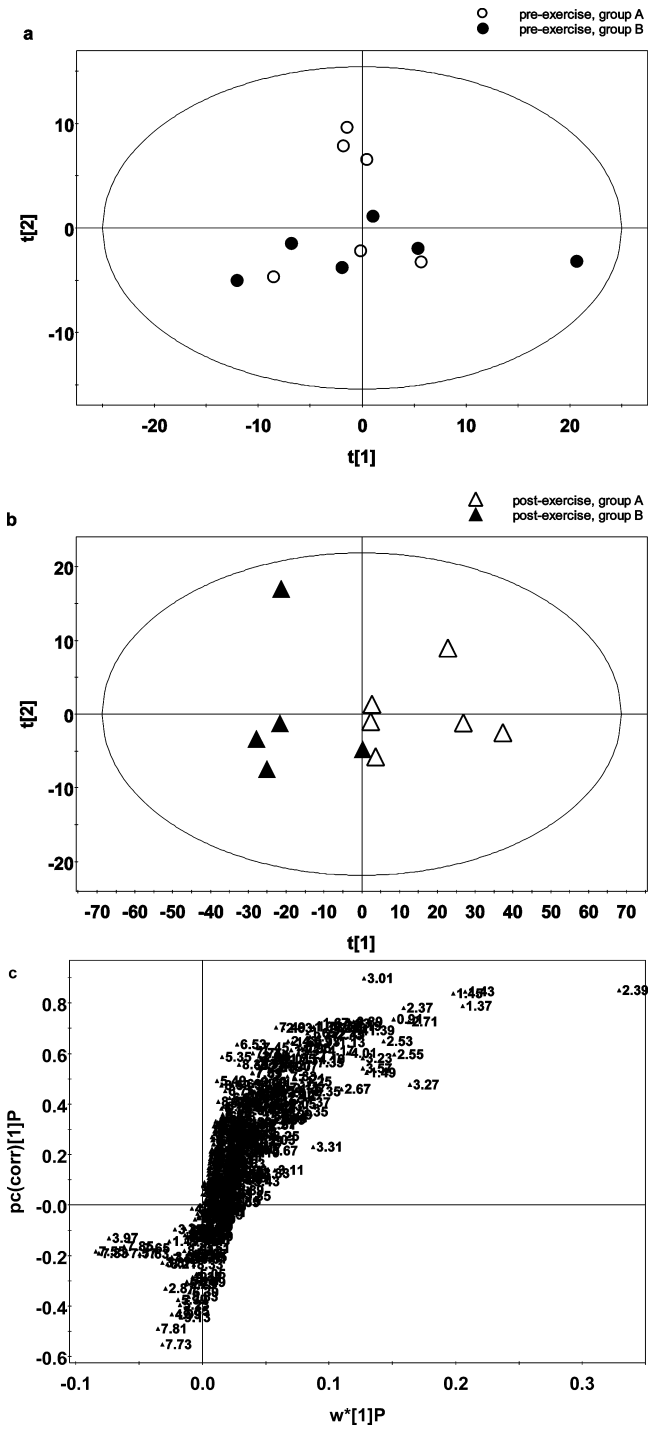

Figure 6. Multivariate statistical analysis of the effect of rest interval duration on the urine metabolome. (a) PCA scores plot of the pre-exercise samples shows no separation of groups A (10 s interval) and B (1 min interval), suggesting equivalence at rest. (b) PCA scores plot of the postexercise samples shows group clustering, suggesting different effects of the two exercise protocols. (c) S-plot of the postexercise samples highlights the differentiators of the groups, with signals on the upper right end being higher in group A and signals on the lower left end being higher in group B.

cal models were built, one including the pre-exercise samples from groups A and B and another including the postexercise samples of the two groups. The groups were not differentiated at rest by PCA (Figure 6a), suggesting that, in addition to being equivalent in terms of age, body mass, and height, they were equivalent in terms of resting metabolic fingerprints. This was confirmed by simple main effect analysis, that is, a pairwise test in the context of the univariate analysis of variance described below, which showed no difference between groups in the pre-exercise values of any metabolite.

In contrast, the differentiation of groups was clear in the model including the postexercise samples (Figure 6b). Lactate, being higher in group A compared to group B, was the major differentiator. PLS-DA did not improve the separation of the pre-exercise samples (no component was calculated), but the postexercise samples of each group were clearly separated (scores plot in Supporting Information Figure S6) in a model that exhibited R2X, R2Y, and Q2 values of 0.686, 0.962, and 0.728, respectively, thus indicating robustness and good predictability. Markers exhibiting higher alteration in group A compared to group B after exercise (S-plot shown in Figure 6c) were, in addition to lactate, 2-hydroxybutyrate, 2-oxoisocaproate, 3-methyl-2-oxovalerate, 2-oxoisovalerate, alanine, pyruvate, citrate, 2-oxoglutarate, and fumarate, along with an unknown metabolite at 3.19 ppm. Metabolites that changed more in group B compared to group A were glycine and hypoxanthine. In all, 12 identified metabolites responded differently to exercise depending on the rest interval between maximal sprints.

3.4. Univariate Analysis. Analysis of variance on the aggregate signals of the 43 metabolites identified in the ^{1}H NMR spectra confirmed most of the findings of PLS-DA, as indicated in Table 2. In particular, the five products of BCAA degradation mentioned above increased after exercise by an average of 14−86% in both groups ($P < 0.05$). Augmentation of 2-oxoisocaproate (the 2-oxoacid, or α-keto acid, of leucine) and 3-methyl-2-oxovalerate (the 2-oxoacid of isoleucine) was higher in group A than in group B ($P < 0.05$).

2-Hydroxybutyrate, derived from methionine in the course of cysteine synthesis, displayed significant main effects of exercise and interval, as well as a significant interaction of exercise and rest interval between repeated sprints (all $P \leq 0.05$). These statistical outcomes were due to the increase with exercise in group A (by 165%) being higher than that in group B (by 58%).

Large increases after exercise were found in the two products of the anaerobic carbohydrate degradation, that is, lactate and pyruvate. In addition, these metabolites displayed a highly significant effect of interval and a highly significant interaction of exercise and interval (all $P < 0.001$). These were due to the postexercise increases in group A being about double those in group B. Thus, lactate increased 64-fold in group A and 30-fold in group B, while pyruvate increased 4-fold in group A and 2-fold in group B.

Amino acids that varied significantly with exercise included alanine, which doubled ($P < 0.001$), and glycine, tryptophan, and histidine, which decreased by 28, 17, and 29%, respectively (all $P < 0.05$). Of the Krebs cycle metabolites, citrate decreased after exercise (by 38%, $P = 0.005$), while 2-oxoglutarate and fumarate increased ($P < 0.01$), the increase being higher in group A than B (27 vs 15% for 2-oxoglutarate and 166 vs 73% for fumarate, both $P < 0.05$). Finally, three other metabolites that changed significantly with exercise according to the ANOVA were trimethylamine *N*-oxide, which decreased by 20% ($P = 0.044$), hypoxanthine, which increased 8-fold ($P < 0.001$), and formate, which decreased by 30% ($P = 0.020$).

4. Discussion

Metabonomic investigation of biological fluids following physical exercise may provide useful information in understanding exercise biochemistry. Urine has not been widely used in exercise metabonomics. In a very recent study Enea et al.[18] detected changes after exercise in the concentration of 11 metabolites by using ^{1}H NMR analysis of urine from female athletes and nonathletes. Most of the remaining reports on exercise metabolomics/metabonomics[11−16,20−22] have investigated metabolite changes in blood serum or plasma.

NMR provides a powerful tool for metabonomic surveys, as it offers direct analysis for a variety of analytes, some of which are difficult to detect with other technologies (e.g., small polar molecules, such as organic acids and sugars). Our results show that physical exercise caused dramatic changes in the urine metabolome; hence, postexercise urine provided very different profiles compared to pre-exercise urine. Even after the exclusion of the lactate signal, PCA alone could differentiate pre- from postexercise urine (whereas in the report by Enea et al.[18] exclusion of urea, creatinine, and lactate resulted in only partial separation). In the present paper, we also investigated the effect of different rest intervals (10 s vs 1 min) between sprints on the urine metabolome. It was intriguing to find that the postexercise samples of the two groups were differentiated. The critical difference between the two exercise sessions is that the 10 s interval allows for hardly any replenishment of immediate energy sources through aerobic reactions, whereas this may be partly accomplished during the 1 min interval.

Most of the energy needed in sprints like the ones employed in the present study is provided by two anaerobic energy systems, the ATP−phosphocreatine system and the lactate system.[30] The increase in the lactate production rate within exercising muscles, resulting from accelerated carbohydrate breakdown, and the concomitant increase in the blood lactate concentration is a common corollary of exercise. Few reports exist regarding the urine lactate response to exercise,[31−33] and these indicate concentrations that are much higher than the corresponding muscle or blood concentrations, similarly to the data of the present study. Thus, finding higher lactate concentrations in the post- compared to the pre-exercise urine samples was not surprising. What was remarkable, however, was the highly significant difference between groups in the postexercise samples. This cannot be explained by a difference in running speed (and, hence, a difference in exercise intensity which is the main determinant of lactate production rate) since the two groups did not differ in running time. However, the difference between groups can be explained by the difference in the duration of the rest interval between repeated sprints. In particular, the very limited time available for phosphocreatine resynthesis (through aerobic ATP resynthesis from ADP and P_i in the mitochondria) between the runs in group A (10 s) may have forced the group's members to enter the second run with less phosphocreatine, less ATP, and more of ATP's degradation products, i.e., P_i, ADP, and AMP. Higher levels of P_i (a substrate in glycogenolysis) and AMP (an allosteric activator of phosphorylase *b*), as well as a lower level of ATP (an inhibitor of phosphorylase *b* activation by AMP), would have resulted in higher acceleration of glycogen breakdown throughout the second run[34] and, hence, higher increases in its products, pyruvate and lactate. Our findings through both the multivariate and univariate analyses are in agreement with this hypothesis, suggesting that the urine concentrations of pyruvate and lactate reflect the amounts produced in the exercising muscles during exercise. These and other effects of exercise and rest interval on the urine metabolome that are discussed below (based primarily on the results of multivariate analysis) are depicted graphically in Figure 7.

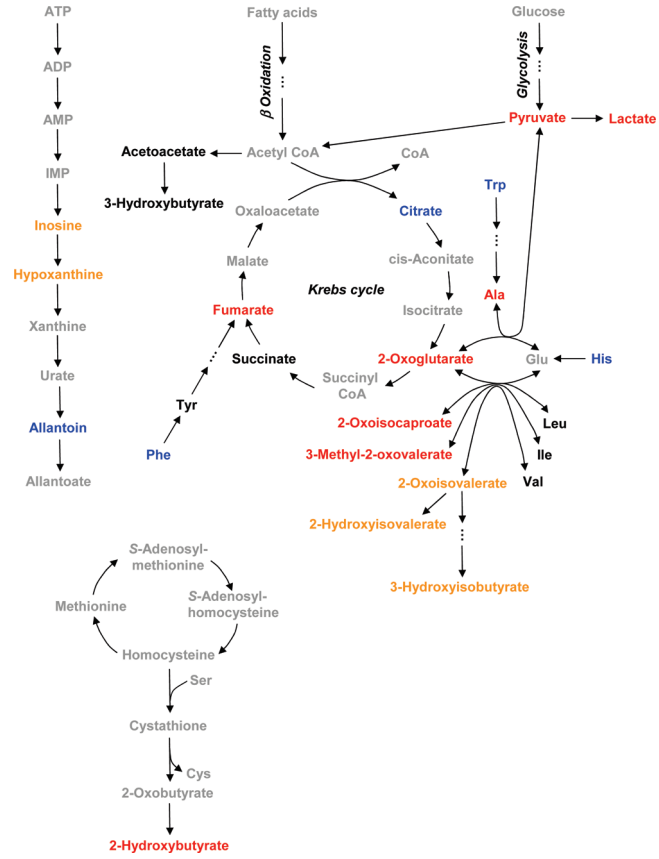

Figure 7. Pathways of ATP, carbohydrate, lipid, and amino acid metabolism with indication of the effect of intermittent sprint training on urine metabolite concentrations through color coding as follows: black, no change; orange, increase; red, higher increase after sprints with a 10 s rest interval compared to sprints with a 1 min rest interval; blue, decrease; gray, metabolites that were not identified or quantified.

A multitude of other metabolites exhibited changes in their urine concentrations after exercise. Inosine and hypoxanthine are products of purine degradation, and their increased concentrations in the postexercise urine samples (especially that of hypoxanthine, which exhibited the largest increase next to lactate) are indicative of the high rate of ATP turnover during sprinting. However, the concentration of allantoin, a product of purine degradation downstream of hypoxanthine, decreased with exercise. In humans, allantoin is produced nonenzymatically through the oxidation of urate by superoxide (a reactive oxygen species) and is further hydrolyzed to allantoate. The decrease in allantoin suggests that the particular exercise protocol employed may not have increased superoxide production. Likewise, the lack of changes in the ketone bodies, acetoacetate and 3-hydroxybutyrate, suggests that there was no effect of sprinting exercise on the metabolism of these products of fatty acid degradation. This is understandable since ketone bodies are formed when there is a shortage of carbohydrates, and such a shortage is not expected to occur during brief exercise.

The increases in alanine and 2-oxoglutarate after exercise and the higher increases in these two metabolites in group A compared to group B are probably the results of the same changes in

pyruvate, since alanine and 2-oxoglutarate are produced from pyruvate and glutamate through transamination. The similar response of fumarate (that is, increase after exercise and higher increase in group A) can be explained by its position downstream of 2-oxoglutarate in the Krebs cycle. The increases in alanine and fumarate after exercise may also be due to increased catabolism of tryptophan and phenylalanine, respectively, since part of the carbon skeleton of the latter two is converted into the former two[35] and since both these aromatic amino acids decreased with exercise in the present study.

Regarding citrate, the metabolite of the Krebs cycle that decreased after exercise, it has been reported that its levels in rat urine decreased along with increasing blood and urinary lactate levels, which were caused by administration of mercuric chloride.[36] The authors hypothesized that this lactic acidosis caused renal tubular acidosis. It is thus possible that the lactic acidosis caused by maximal exercise in the present study may have compromised renal function. Attesting to this hypothesis are the findings of reduced glycine and hippurate after exercise, both of which have been described as signs of reversible renal malfunction.[37]

The finding of exercise-induced increases in all five products of BCAA degradation that we were able to identify is in agreement with the prevailing notion in the literature that BCAA degradation increases in muscle with exercise.[38] Two relevant observations are worthy of mention. First, the postexercise urine samples resemble the profile of maple syrup urine disease[38] in which there is a deficiency of branched-chain 2-oxoacid dehydrogenase (BCOAD), resulting in a buildup of branched-chain 2-oxoacids (2-oxoisovalerate, 2-oxoisocaproate, and 3-methyl-2-oxovalerate) that cannot be oxidized, as well as of 2-hydroxyisovalerate (produced by reduction of 2-oxoisovalerate). However, the increase of these metabolites in the urine after exercise should not be interpreted as a defect or inhibition of BCOAD, which, on the contrary, is activated by exercise.[39] Attesting to this is the increase in 3-hydroxyisobutyrate, which lies past the reaction catalyzed by BCOAD in the pathway of valine catabolism. Rather, the increase in branched-chain 2-oxoacids seems to reflect their overproduction through transamination of BCAAs which, in turn, exhibit increased turnover during exercise as a result of increased proteolysis in the visceral area and muscle.[40] Second, the products of leucine and isoleucine degradation (2-oxoisocaproate and 3-methyl-2-oxovalerate, respectively) but not those of valine degradation (2-oxoisovalerate, 2-hydroxyisovalerate, and 3-hydroxyisobutyrate) increased more in group A than in group B, suggesting that the three BCAAs may not respond uniformly to exercise.

Of interest is the exercise-induced increase in 2-hydroxybutyrate and its higher postexercise concentration in group A compared to group B. 2-Hydroxybutyrate is produced in mammalian tissues (principally hepatic) that synthesize glutathione. Oxidative stress can dramatically increase the rate of hepatic glutathione synthesis. Under such conditions, supplies of cysteine for glutathione synthesis become limiting, so homocysteine is diverted from the transmethylation pathway forming methionine into the transsulfuration pathway forming cystathionine. 2-Oxobutyrate is released when cystathionine is cleaved to cysteine. 2-Oxobutyrate is then reduced by lactate dehydrogenase to 2-hydroxybutyrate.[41] It has been reported that an increased [NADH]/[NAD^+] ratio is the most important factor for the production of 2-hydroxybutyrate,[42] and this observation may explain the higher postexercise 2-hydroxybutyrate concentration in group A, since a higher glycolytic rate as a result of the shorter rest interval, as evidenced by the higher pyruvate concentration, would result in a higher rate of conversion of NAD^+ to NADH.

Formate is derived from methanol which is either inhaled or ingested (mainly in the form of methyl esters) from foods such as fresh fruits, fruit juices, certain vegetables, and the artificial sweetener, aspartame. Methanol is converted to formaldehyde by methanol dehydrogenase, and formaldehyde is subsequently converted to formate by formaldehyde dehydrogenase.[43] Both dehydrogenations require NAD^+ as the reducing agent. Thus, it is possible that the shortage of NAD^+ as a result of high-intensity exercise, as explained above, may have caused a decrease in the rate of formate production. This may explain the lower formate concentration in the post- compared to the pre-exercise urine samples.

1-Methylhistidine and 3-methylhistidine are two amino acids that are considered as markers of meat consumption[44] and muscle protein breakdown.[40] The lack of changes in their urinary levels after short-term exercise in the present study is in accordance with their dietary origin, on one hand, and suggests that the exercise protocol employed did not change the rate of muscle protein breakdown.

5. Conclusions

The present study illustrates the utility of holistic analytical methods in the study of exercise metabolism. ^{1}H NMR-based metabonomics provided useful information for the understanding of metabolic changes induced by specific training schedules. Urine samples obtained after exercise protocols differing in as little as the rest interval between repeated sprints, not the sprints themselves, can be classified and safely predicted even when applying nonsupervised statistical methods of analysis. In this way, important biomolecules involved in exercise biochemistry can be identified and further studied. Separation of pre- from postexercise samples was assigned mainly to lactate, pyruvate, hypoxanthine, compounds of the Krebs cycle, amino acids, products of BCAA catabolism, 2-hydroxybutyrate, and hippurate. Most of these metabolites increased in urine with exercise and have been described to also increase in muscle with exercise. Separation of the 10 s from the 1 min rest interval was assigned mainly to lactate, pyruvate, alanine, compounds of the Krebs cycle, 2-oxoacids of BCAA, and 2-hydroxybutyrate. All of these metabolites increased more with the short compared to the long interval, thus supporting the hypothesis that the former elicited greater metabolic disturbances than the latter as a result of the very limited time available for recovery. The facts that such methodology can be applied to urine, a biological material readily available and noninvasively obtained, and that urine reflects many of the exercise-induced changes occurring in muscle are additional advantages.

Acknowledgment. The authors wish to thank Prof. Ian Wilson, AstraZeneca, U.K., for useful discussion and for reading the manuscript. H. Gika's work is funded by Marie Curie ERG 202132 Reintegration grant from the European Committee. A. Pechlivanis and H. Gika acknowledge the provision of laboratory space for computational work from the Laboratory of Forensic Medicine and Toxicology, Medical School, Aristotle University Thessaloniki. Sarantos Kostidis activity has been carried out with the financial support of the

Commission of the European Community, Framework Program 7, specific RTD program "Research Potential", NATFORCE (Grant Agreement no: 206570). It does not necessarily reflect the Commissions views and in no way anticipates its future policy in this area. This work was supported in part by a donation from the Bodossakis foundation.

Supporting Information Available: Tables S1 and S2 and Figures S1−S6. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Roepstorff, C.; Halberg, N.; Hillig, T.; Saha, A. K.; Ruderman, N. B.; Wojtaszewski, J. F.; Richter, E. A.; Kiens, B. Malonyl-CoA and carnitine in regulation of fat oxidation in human skeletal muscle during exercise Am. *J. Physiol. Endocrinol. Metab.* **2005**, *288*, 133–142.

(2) Mikami, T.; Kitagawa, J. Intense exercise induces the degradation of adenine nucleotide and purine nucleotide synthesis via de novo pathway in the rat liver. *Eur. J. Appl. Physiol.* **2006**, *96*, 543–550.

(3) Nicholson, J. K.; Lindon, J. C.; Holmes, E. Metabonomics': Understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* **1999**, *29*, 1181–1189.

(4) Nicholson, J. K.; Lindon, J. C. Systems biology: Metabonomics. *Nature* **2008**, *455*, 1054–1056.

(5) Nicholson, J. K.; Connelly, J.; Lindon, J. C.; Holmes, E. Metabonomics: a platform for studying drug toxicity and gene function. *Nat. Rev. Drug Discovery* **2002**, *1*, 253–258.

(6) Theodoridis, G.; Gika, H.; Wilson, I. D. LC-MS-based methodology for global metabolite profiling in metabonomics/metabolomics. *TrAC, Trends Anal. Chem.* **2008**, *27*, 251–260.

(7) Lenz, E. M.; Wilson, I. D. Analytical Strategies in Metabonomics. *J. Proteome Res.* **2007**, *6*, 443–458.

(8) Lindon, J.; Nicholson, J. K.; Holmes, E., Eds. *The Handbook of Metabonomics and Metabolomics*; Elsevier: Amsterdam, The Netherlands, 2007.

(9) Dumas, M. E.; Maibaum, E. C.; Teague, C.; Ueshima, H.; Zhou, B.; Lindon, J. C.; Nicholson, J. K.; Stamler, J.; Elliot, P.; Chan, Q.; Holmes, E. Assessment of Analytical Reproducibility of 1H NMR Spectroscopy Based Metabonomics for Large-Scale Epidemiological Research: the INTERMAP Study. *Anal. Chem.* **2006**, *78*, 2199–2208.

(10) Bertram, H. C.; Malmendal, A.; Petersen, B. O.; Madsen, J. C.; Pedersen, H.; Nielsen, N. C.; Hoppe, C.; Molgaard, C.; Michaelsen, K. F.; Duus, J. O. Effect of Magnetic Field Strength on NMR-Based Metabonomic Human Urine Data. Comparative Study of 250, 400, 500, and 800 MHz. *Anal. Chem.* **2007**, *79*, 7110–7115.

(11) Pohjanen, E.; Thysell, E.; Jonsson, P.; Eklund, C.; Silfver, A.; Carlsson, I. B.; Lundgren, K.; Moritz, T.; Svensson, M. B.; Antti, H. A multivariate screening strategy for investigating metabolic effects of strenuous physical exercise in human serum. *J. Proteome Res.* **2007**, *6*, 2113–2120.

(12) Lewis, G. D.; Farrell, L.; Wood, M. J.; Martinovic, M.; Arany, Z.; Rowe, G. C.; Souza, A.; Cheng, S.; McCabe, E. L.; Yang, E.; Shi, X.; Deo, R.; Roth, F. P.; Asnani, A.; Rhee, E. P.; Systrom, D. M.; Semigran, M. J.; Vasan, R. S.; Carr, S. A.; Wang, T. J.; Sabatine, M. S.; Clish, C. B.; Gerszten, R. E. Metabolic signatures of exercise in human plasma. *Sci. Transl. Med.* **2010**, *2*, 33–37.

(13) Yan, B.; Jiye, A.; Wang, G.; Lu, H.; Huang, X.; Liu, Y.; Zha, W.; Hao, H.; Zhang, Y.; Liu, L.; Gu, S.; Huang, Q.; Zheng, Y.; Sun, J. Metabolomic investigation into variation of endogenous metabolites in professional athletes subject to strength-endurance training. *J. Appl. Physiol.* **2009**, *106*, 531–538.

(14) Kirwan, G. M.; Coffey, V. G.; Niere, J. O.; Hawley, J. A.; Adams, M. J. Spectroscopic correlation analysis of NMR-based metabonomics in exercise science. *Anal. Chim. Acta* **2009**, *652*, 173–179.

(15) Chorell, E.; Moritz, T.; Branth, S.; Antti, H.; Svensson, M. B. Predictive Metabolomics Evaluation of Nutrition-Modulated Metabolic Stress Responses in Human Blood Serum During the Early Recovery Phase of Strenuous Physical Exercise. *J. Proteome Res.* **2009**, *8*, 2966–2977.

(16) Miccheli, A.; Marini, F.; Capuani, G.; Miccheli, A. T.; Delfini, M.; Di Cocco, M. E.; Puccetti, C.; Paci, M.; Rizzo, M.; Spataro, A. The Influence of a Sports Drink on the Postexercise Metabolism of Elite Athletes as Investigated by NMR-Based Metabolomics. *J. Am. Coll. Nutr.* **2009**, *28*, 553–564.

(17) Huang, C. C.; Lin, W. T.; Hsu, F. L.; Tsai, P. W.; Hou, C. C. Metabolomics investigation of exercise-modulated changes in metabolism in rat liver after exhaustive and endurance exercises. *Eur. J. Appl. Physiol.* **2010**, *108*, 557–566.

(18) Enea, C.; Seguin, F.; Petitpas-Mulliez, J.; Boildieu, N.; Boisseau, N.; Delpech, N.; Diaz, V.; Eugene, M.; Dugué, B. 1H NMR-based metabolomics approach for exploring urinary metabolome modifications after acute and chronic physical exercise. *Anal. Bioanal. Chem.* **2010**, *396*, 1167–1176.

(19) Lee, R.; West, D.; Phillips, S. M.; Britz-McKibbin, P. Differential Metabolomics for Quantitative Assessment of Oxidative Stress with Strenuous Exercise and Nutritional Intervention: Thiol-Specific Regulation of Cellular Metabolism with N-Acetyl-l-Cysteine Pretreatment. *Anal. Chem.* **2010**, *82*, 2959–2968.

(20) Barba, I.; de León, G.; Martín, E.; Cuevas, A.; Aguade, S.; Candell-Riera, J.; Barrabés, J. A.; Garcia-Dorado, D. Nuclear magnetic resonance-based metabolomics predicts exercise-induced ischemia in patients with suspected coronary artery disease. *Magn. Reson. Med.* **2008**, *60*, 27–32.

(21) Coolen, S. A.; Daykin, C. A.; van Duynhoven, J. P. M.; van Dorsten, F. A.; Wulfert, F.; Mathot, J.; Scheltinga, M. R.; Stroosma, O.; Vader, H.; Wijnen, M. H. Measurement of ischaemia-reperfusion in patients with intermittent claudication using NMR-based metabonomics. *NMR Biomed.* **2008**, *21*, 686–695.

(22) Kuhl, J.; Moritz, T.; Wagner, H.; Stenlund, H.; Lundgren, K.; Båvenholm, P.; Efendic, S.; Norstedt, G.; Tollet-Egnell, P. Metabolomics as a tool to evaluate exercise-induced improvements in insulin sensitivity. *Metabolomics* **2008**, *4*, 273–282.

(23) Bax, A.; Davis, D. G. MLEV-17-based two-dimensional homonuclear magnetization transfer spectroscopy. *J. Magn. Reson.* **1985**, *65*, 355–360.

(24) Holmes, E.; Foxal, P. J. D.; Nicholson, J. K.; Neild, G. H.; Brown, S. M.; Beddell, C. R.; Sweatman, B. C.; Rahr, E.; Lindon, J. C.; Spraul, M.; Neidig, P. Automatic data reduction and pattern recognition methods for analysis of 1H NMR spectra of human urine from normal and pathological states. *Anal. Biochem.* **1994**, *220*, 284–296.

(25) Lindon, J. C.; Nicholson, J. K.; Everett, J. R. NMR spectroscopy of biofluids. *Ann. Rep. NMR Spectrosc.* **1999**, *38*, 2–87.

(26) Holmes, E.; Foxall, P. J. D.; Spraul, M.; Farrant, R. D.; Nicholson, J. K.; Lindon, J. C. 750 MHz 1H NMR spectroscopy characterisation of the complex metabolic pattern of urine from patients with inborn errors of metabolism: 2-hydroxyglutaric aciduria and maple syrup urine disease. *J. Pharm. Biomed. Anal* **1997**, *15*, 1647–1659.

(27) SIMCA-P 12, *User Guide and Tutorial*; Kinnelon, NJ: USA, 2008; pp 262.

(28) Cloarec, O.; Dumas, M. E.; Craig, A.; Barton, R. H.; Trygg, J.; Hudson, J.; Blancher, C.; Gauguier, D.; Lindon, J. C.; Holmes, E.; Nicholson, J. K. Statistical Total Correlation Spectroscopy: An Exploratory Approach for Latent Biomarker Identification from Metabolic 1H NMR Data Sets. *Anal. Chem.* **2005**, *77*, 1282–1289.

(29) Trygg, J.; Holmes, E.; Lundstedt, T. Chemometrics in Metabonomics. *J. Proteome Res.* **2007**, *6*, 469–479.

(30) Gastin, P. B. Energy System Interaction and Relative Contribution During Maximal Exercise. *Sports Med.* **2001**, *31*, 725–741.

(31) Liljestrand, S. H.; Wilson, D. W. The excretion of lactic acid in the urine after muscular exercise. *J. Biol. Chem.* **1925**, *65*, 773–782.

(32) Johnson, R. E.; Edwards, H. T. Lactate and pyruvate in blood and urine after exercise. *J. Biol. Chem.* **1937**, *118*, 427–432.

(33) Judge, M. A.; van Eys, J. Excretion of D-lactic acid by humans. *J. Nutr.* **1962**, *76*, 310–313.

(34) Greenhaff, P. L.; Hultman, E.; Harris, R. C. Carbohydrate metabolism. In *Principles of Exercise Biochemistry*; Poortmans, J. R., Ed.; Karger: Basel, Switserland, 2004; pp 108−151.

(35) Berg, J. M.; Tymoczko, J. L.; Stryer, L. *Biochemistry*; Freeman: New York, NY, 2002; pp 654−655.

(36) Nicholson, J. K.; Timbrell, J. A.; Sadler, P. Proton NMR spectra of urine as indicators of renal damage mercury-induced nephrotoxicity in rats. *J. Mol. Pharmacol.* **1985**, *27*, 644–651.

(37) Bairaktari, E.; Seferiadis, K.; Liamis, G.; Psihogios, N.; Tsolas, O.; Elisaf, M. Rhabdomyolysis-related Renal Tubular Damage

Studied by Proton Nuclear Magnetic Resonance Spectroscopy of Urine. *Clin. Chem.* **2002**, *48*, 1106–1109.
(38) Mougios, V. Exercise Biochemistry; *Human Kinetics*: Champaign IL, 2006; pp 216−218.
(39) Engelke, U.; Moolenaar, S.; Hoenderop, S.; Morava, E.; van der Graaf, M.; Heerschap, A.; Wevers, R. *Handbook of ^{1}H-NMR spectroscopy in inborn errors of metabolism: body fluid NMR spectrum and in vivo MR spectroscopy*; SPS Verlagsgesellschaft: Heilbronn, Germany, 2007; p 65.
(40) Poortmans, J. R. Protein metabolism. In *Principles of Exercise Biochemistry*; Poortmans, J. R., Ed.; Karger: Basel, Switzerland, 2004; pp 227−278.
(41) Pettersen, J. E.; Landaas, S.; Eldjarn, L. The occurrence of 2-hydroxybutyric acid in urine from patients with lactic acidosis. *Clin. Chim. Acta* **1973**, *48*, 213–219.
(42) Landaas, S.; Pettersen, J. E. The occurrence of 2-hydroxyisovaleric acid in patients with lactic acidosis and ketoacidosis. *Scand. J. Clin. Lab. Invest.* **1975**, *35*, 259–266.
(43) Ernstgård, L.; Shibata, E.; Johanson, G. Uptake and Disposition of Inhaled Methanol Vapor in Humans. *Toxicol. Sci.* **2005**, *88*, 30–38.
(44) Dragsted, L. O. Biomarkers of meat intake and the application of nutrigenomics. *Meat Sci.* **2010**, *84*, 301–307.

PR100684T