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^{1}H NMR study of the effects of sample contamination in the metabolomic analysis of mouse urine

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Abstract

Nuclear magnetic resonance (NMR) spectroscopy was used to evaluate and optimize the strategy for collecting mouse urine samples. A series of normal urine samples and those mixed with folate-deficient food, turkey or mouse fecal particles were analyzed using principal component analysis (PCA). The metabolic profile of urine mixed with folate-deficient food was found to be extremely different than that of clean urine. Changes in the urine composition caused by mixing with turkey or feces are relatively small as judged by the output of PCA. As a result, turkey may be considered as an applicable food source for obtaining uncontaminated urine samples for metabolomics-based research.

Keywords: Nuclear magnetic resonance (NMR); Metabolomics; Mouse urine; Principal component analysis (PCA); Diet

1. Introduction

The combination of high-resolution NMR spectroscopy with multivariate statistics is a powerful approach for the study of metabolomics [1]. ^{1}H NMR analysis is widely used to identify and quantify the metabolites in biofluid samples because it can simultaneously display resonance peaks resulting from hundreds of metabolites, and requires little or no sample preparation. In combination with multivariate statistics [2,3], such as principle component analysis (PCA), NMR data can produce a metabolic profile for further understanding changes in metabolism or for early disease detection [4–9]. Pretreatment of the data, either by scaling [10], noise reduction [11] or selective spectroscopic methods [12] can be used to reduce the variability introduced by the samples. Alternatively, combined analytical approaches such as NMR and MS [13–16] can improve the analysis of complex samples, while uninteresting features can be eliminated by statistical methods such as orthogonal signal correction [17].

However, a related but less discussed problem involves sample contamination. In particular, collecting animal samples under highly controlled conditions continues to be a problem which can complicate the useful data in such a way that even the most sophisticated spectroscopic and statistical methods have trouble recovering. It is obvious, therefore, that an important goal of metabolomics-based research is to obtain a clean and reliable set of samples in order to focus the analysis on more important metabolic variations that indicate disease, toxicity, etc. This is particularly a problem for studies involving mice since they do not produce much urine or other biofluids compared to larger animals. Nevertheless, their study provides an important avenue for metabolomics research since there have been so many disease models created in transgenic mice during the past 20 years [18–20]. In order to improve sample treatment, centrifugation has been used to remove food or fecal particles that often get inadvertently mixed with the urine and contaminate the samples [21]. However, as we show below, this treatment may be insufficient for food contamination. In the present study, ^{1}H NMR spectroscopy has been used to evaluate the effect of food or fecal particle contamination during sample collection, which is an important step in metabolomics research. By preparing urine samples in different methods prior to NMR analysis, it is found

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Table 1
Labels and volumes of urine samples collected twice a week during four continuous weeks

Week	Sampling period	Volume (ml) and labels			
		M053	M054	M055	M056
1	1	3.03[a] **(1)**[b]	2.94[a] **(2)**	2.62[a] **(3)**	4.45[a] **(4)**
	2	2.05[a] **(5)**	3.66[a] **(6)**	4.45[a] **(7)**	2.88 **(8)**
2	3	2.20 **(9)**	1.66[a] **(10)**	2.27[a] **(11)**	1.75 **(12)**
	4	0.75[a] **(13)**	4.05[a] **(14)**	3.29 **(15)**	1.69 **(16)**
3	5	8.48 **(17)**	1.93 **(18)**	2.85 **(19)**	3.13 **(20)**
	6	7.94 **(21)**	5.21 **(22)**	7.39 **(23)**	1.49[a] **(24)**
4	7	1.55[a] **(25)**	1.07 **(26)**	6.73 **(27)**	1.87 **(28)**
	8	3.13[a] **(29)**	1.64[a] **(30)**	1.40 **(31)**	1.28 **(32)**

[a] Food or fecal particles visible in urine sample.
[b] Sample indices indicated in bold.

Table 2
Sample preparations used in the mixing studies. Higher volume samples were used for multiple sample preparations

Preparation	Additive	Labels			
		M053	M054	M055	M056
Vortex	Fecal particles	17[a] **(33)**[b]	22 **(34)**	23 **(35)**	8 **(36)**
	Folate-deficient food	17 **(37)**	22 **(38)**	23 **(39)**	8 **(40)**
	Turkey	17 **(41)**	22 **(42)**	23 **(43)**	28 **(44)**
Non-vortex	Folate-deficient food	9 **(45)**	18 **(46)**	15 **(47)**	12 **(48)**
	Turkey	29 **(49)**	2 **(50)**	19 **(51)**	16 **(52)**
Incubation	Fecal particles	1 **(53)**	6 **(54)**	23 **(55)**	20 **(56)**
	Folate-deficient food	17 **(57)**	14 **(58)**	27 **(59)**	4 **(60)**
	Turkey	21 **(61)**	14 **(62)**	27 **(63)**	4 **(64)**

[a] Sample indices from Table 1.
[b] Sample indices after preparation, indicated in bold.

that folate-deficient food particles mixed in urine have a large effect on the urine composition as can be seen in the results of PCA, while the effect of turkey or feces contamination is much smaller. Thus, a method for reducing the influence of external contaminants when collecting mouse urine is presented.

2. Materials and methods

2.1. Collection of urine samples

Urine samples were obtained from four CD-1 male mice (Harlan, Indianapolis, IN) at similar age (75 days) during four consecutive weeks. As the preparation for the current study, the four mice (labeled M053–M056) were fed in vented cages for 1 week with folate-deficient food (Teklad Folic Acid Deficient Diet TD00434, Harlan Teklad, Madison, WI). This food was chosen because folic acid-based therapies are the focus of some cancer studies [22] and the presence of excess folic acid may reduce the effectiveness of this therapy. After the first week, the mice were housed in separate metabolic cages with free access to water as well as turkey meat (Carl Buddig, Homewood, IL). BASi metabolic cages were used and are described in the supplementary information Fig. S1. Urine was collected during a 24-h collection period using a refrigerated collection device, after which the mice were removed from the metabolic cages and placed in vented cages with free access to folate-deficient food. Urine was collected twice a week from each mouse by repeating the 24-h urine collection procedure. In total, 32 untreated urine samples with volumes varying from 0.75 to 8.48 ml were obtained after 4 weeks and stored at −80 °C until NMR analysis was performed. After thawing the samples, but prior to the NMR analysis, 0.1% sodium azide was added to preserve the urine samples. Table 1 shows detailed information about the volumes and timing of urine samples collected for the present study.

2.2. Urinary treatment: vortex, non-vortex and incubation procedures

Three sample-mixing procedures were used to investigate contamination. First, 1 ml of urine from each mouse was vortexed for 30 s with 0.03 g of small turkey slices, two mouse fecal particles (about 0.03 g total), or 0.03 g of ground folate-deficient food. In total, 12 samples were prepared using this method, stored at 5 °C for 12 h then centrifuged at 5000 rpm for 3 min to remove the particles before ^{1}H NMR analysis. An additional eight samples were prepared by mixing 1 ml of urine from each mouse with 0.03 g of small turkey slices or 0.03 g of lump folate-deficient food, directly stored at 5 °C for 12 h, and then centrifuged by the same procedure. Finally, an incubation preparation was used in which 12 one-ml samples had either 0.03 g of small turkey slices, two mouse fecal particles (about 0.03 g), or 0.03 g of folate-deficient food added to them. These 12 samples were stored at room temperature (about 20 °C) for 12 h before centrifugation at 5000 rpm for 3 min. Table 2 describes additives to urine samples and labels after each preparation.

Additional samples were prepared to study the extraction of metabolites from food and feces. For the extraction experiments, samples were prepared by mixing 0.03 g turkey, folate-deficient food or mouse feces with 1 ml of deionized water and then stored at 5 °C for 1 and 24 h. These six samples were then centrifuged to remove insoluble particles for further NMR analysis.

2.3. 1D ^{1}H NMR spectroscopy and PCA analysis

For 1D ^{1}H NMR measurements, 300 μL of each sample (from the three urinary treatments and extraction experiments) was mixed with 300 μL of phosphate buffer solution (K_2HPO_4, KH_2PO_4, 0.5/0.5 M) to stabilize the pH. A 150 μL 3 mM solution of 3-(trimethylsilyl) propionic-(2,2,3,3-d_4) acid sodium salt (TSP) in D_2O was then added as a frequency standard. A 580 μL aliquot of this mixture was transferred to a 5 mm standard NMR tube, and the ^{1}H NMR spectra were then acquired using a Bruker DRX 500 MHz spectrometer equipped with a room temperature HCN probe. The water peak was suppressed using solvent presaturation and the 1D NOESY pulse sequence. Thirty-two transients were collected resulting in 16k data points for each spectrum. Before Fourier transformation, an exponen-

tial weighting function corresponding to 0.3 Hz line broadening was applied to the free induction decay (FID).

After phasing and baseline correction using Bruker's XWIN-NMR software, each spectrum was reduced to 1000 frequency buckets of equal width in order to reduce the matrix size needed for PCA and to remove the effect of small changes in chemical shift due to pH or ion concentration variations. The 4.5–6 ppm spectral region was removed to eliminate urea and residual water peaks. The integrated spectrum was then normalized, and the data were imported into Pirouette software (v. 3.11; InfoMetrix, Woodinville, WA) for PCA.

3. Results and discussion

3.1. ^{1}H NMR spectral study

NMR spectra for untreated urine, and urine treated with folate-deficient food, fecal particles and turkey meat are shown in Figs. 1–3, respectively. The aliphatic region in the spectra of the untreated urine samples are dominated by peaks from creatine (3.94 and 3.04 ppm), trimethylamine N-oxide (TMAO) (3.27 ppm), taurine (3.43 and 3.26 ppm), creatinine (4.08 and 3.05 ppm), and glucose (3.83, 3.76 and 3.42 ppm). Visible peaks from formate (8.46 ppm), tyrosine (7.2 ppm), and phenylacetylglycine (PAG) (7.42, 7.35, 3.75 and 3.67 ppm) are distributed in the aromatic region of the spectra of the untreated urine samples. The assignments are based on literature values and previous work in NMR-based metabolomics [21,23–26].

In general, the collection of pure mouse urine completely free of contamination is challenging. Although filters were added to the caging apparatus, 15 out of 32 untreated samples contained visible particles. Another event worth mentioning is that the volumes of five untreated samples were extremely large compared to the rest of the samples. It seems that some mice spilled water into the samples as they consumed water, which made the suppression of water peaks for these samples less effective under the same experimental conditions.

Fig. 1 provides evidence of the significant changes in the concentration of numerous metabolites caused by mixing urine with folate-deficient food. This situation was observed for all three preparation methods. In particular, glucose, taurine and creatine are observed to change dramatically. Evidently, sugar, amino acids and other metabolites, which are the major components in the folate-deficient mouse food, dissolve readily in urine as reflected in the NMR spectra. The changes are most easily observed for peaks located in the 0.0–4.0 ppm spectral region while there is no significant increase in the aromatic region. It is also observed that the spectral contamination is worst for the vortex mixing preparation.

In contrast to the data presented in Fig. 1, mouse fecal particles appear to introduce very few significant changes to the NMR spectra, as seen in Fig. 2. Some changes, most noticeably an increase in the intensities of the acetate and lactate peaks (1.95 and 1.33 ppm, respectively), are easily visible, while the increase in creatinine and creatine concentrations is more subtle. After mixing mouse urine with turkey meat, the intensities of acetate, lactate, glucose, creatine and creatinine are somewhat

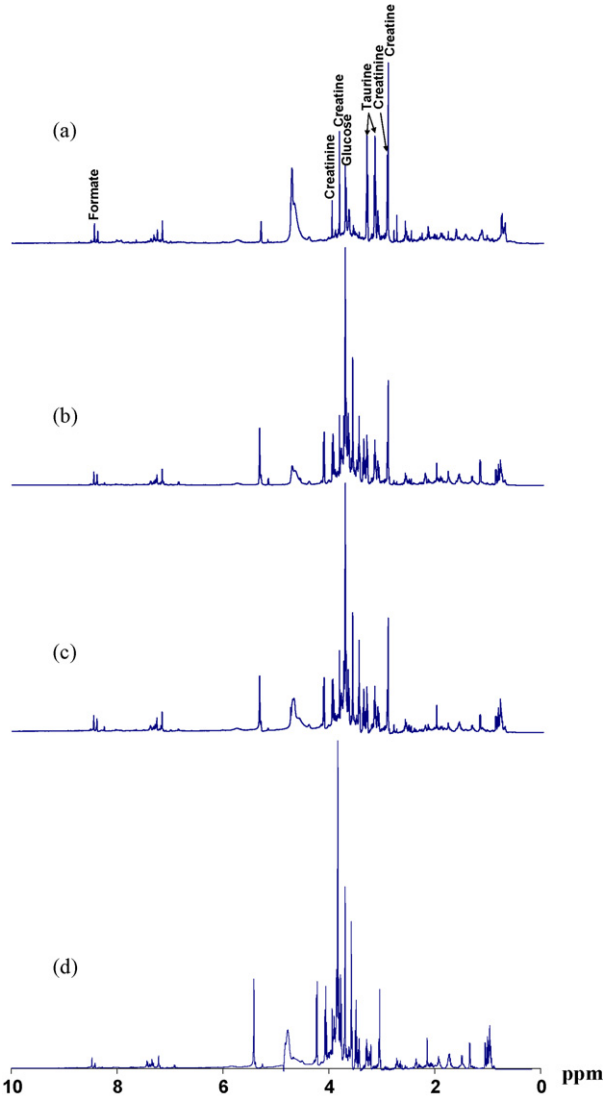

Fig. 1. Typical ^{1}H NMR spectra of mouse urine samples mixed with folate-deficient food: (a) untreated sample; (b) sample with vortex preparation; (c) sample with non-vortex preparation; (d) sample with incubation preparation.

elevated in Fig. 3, and again the variation in the aromatic region is less than that for the aliphatic region.

The results above were confirmed by the NMR spectra of extracts of folate-deficient mouse food, fecal particles, and turkey meat as shown in the Figs. S2–S4, respectively. Panels a and b in these figures represent the NMR spectra of extracts after 1 and 24 h incubation, respectively. It is clear in Fig. S2 that peaks from glucose, methionine (2.14 ppm), alanine (1.48 ppm), lactate and amino acids (0.8–1.0 ppm) appear in the aliphatic regions. Compared with Fig. S2, only relatively small peaks from lactate and acetate can be identified in the fecal extracts (Fig. S3) using the same NMR acquisition paprameters. There are more peaks visible in the NMR spectra of the turkey extracts shown in the Fig. S4 than for the fecal extracts. Glucose, creatine and lactate could be identified in the spectra shown in Fig. S4. Furthermore, more peaks were found in the spectrum, shown in Fig. S4b, than that of Fig. S4a. This may be because more solutes from turkey can dissolve in water during the extra incuba-

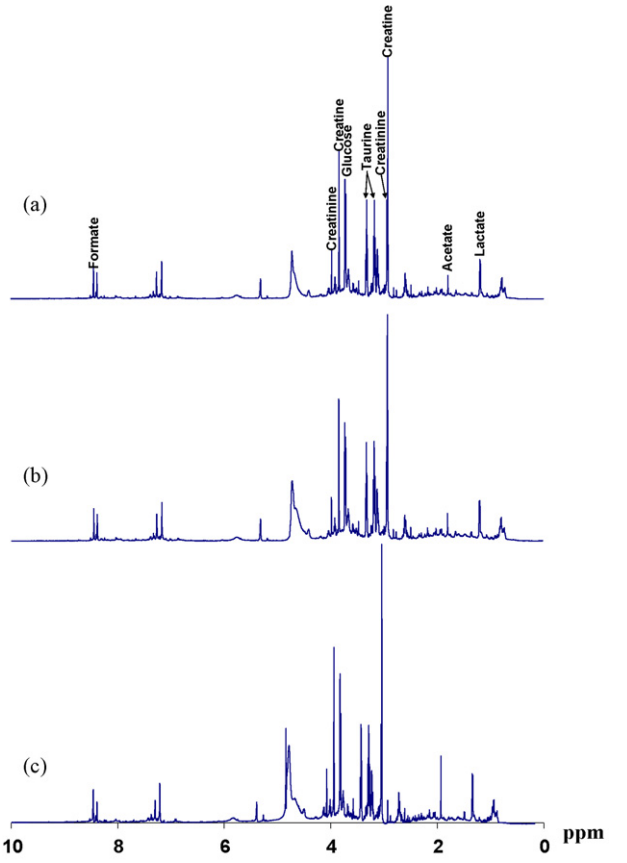

Fig. 2. Typical ^{1}H NMR spectra of mouse urine samples mixed with mouse feces: (a) untreated sample; (b) sample with vortex preparation; (c) sample with incubation preparation.

tion time. Thus, extraction experiments confirm the results that folate-deficient food may cause larger variations in the NMR spectrum than turkey or feces.

3.2. PCA results

In order to extract more detailed metabolic information beyond visual inspection, PCA was performed to study the effect of mixing urine with food or fecal particles using the three different preparations. PCA was first used to evaluate the variations in urinary composition of the untreated 32 urine samples that might be due to dependencies on individual mice, time of collection, or contamination by visible particles. Score plots (Fig. 4) using the first two PCs were used to present a 2D representation of variations among the spectra. Fig. 4a compares the PCA of NMR spectra from different mice. Though there is no obvious separation, there is a slight classification between the cluster of M053 and M056 data and the cluster of M054 and M055 data, which indicates that the metabolism of an indiviual mouse has a small effect on the metabolic profile of urine even when they are treated identically. In Fig. 4b, there is no noticeable classification in terms of the time of collection. In Fig. 4c, there is also no significant difference between urine samples that were collected with or without visual particles. The sample volume does not appear to be a factor in causing any classification, as can be

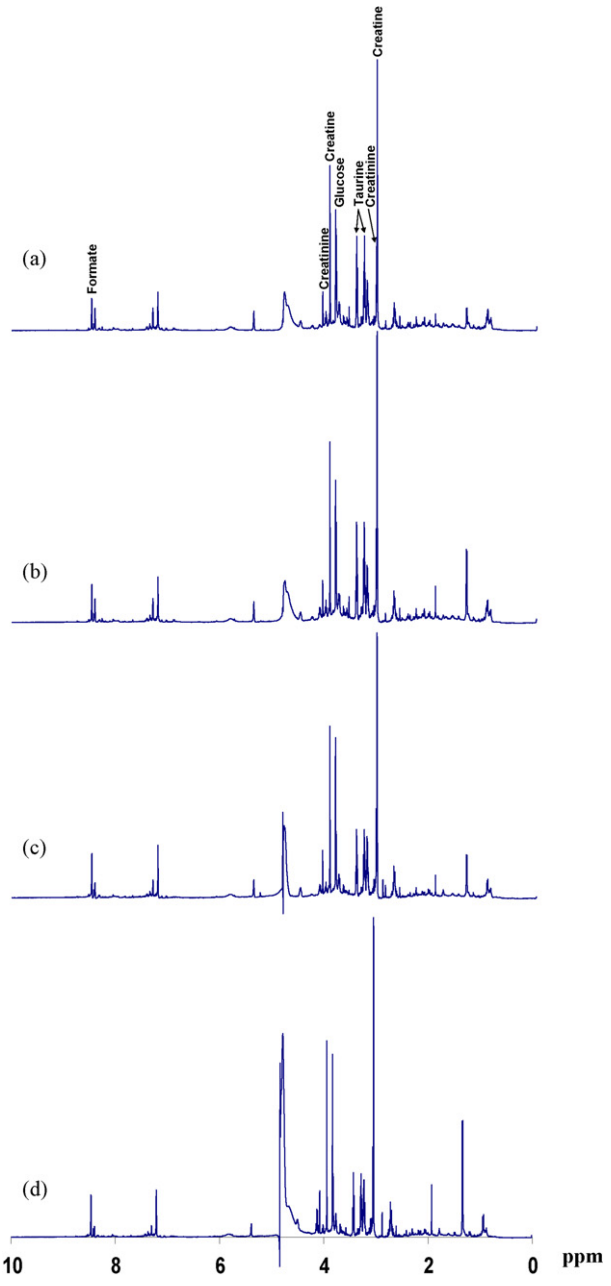

Fig. 3. Typical ^{1}H NMR spectra of mouse urine samples mixed with turkey: (a) untreated sample; (b) sample with vortex preparation; (c) sample with non-vortex preparation; (d) sample with incubation preparation.

seen in Fig. 4c, in which the numbered data points indicate high-volume samples. This result is due to the fact that the integrated spectral intensities were normalized. In terms of the loading plot, compounds in the aliphatic region of the NMR spectra dominate the plot for the 32 untreated samples. In short, these 32 untreated samples are basically the same according to NMR analysis and PCA. It is reassuring that the NMR results indicate that the current protocol provides a reliable method for sample collection. However, it is desirable to reduce the variability of the samples as much as possible.

Considering that there might be some differences resulting from possible contamination of individual samples, the fol-

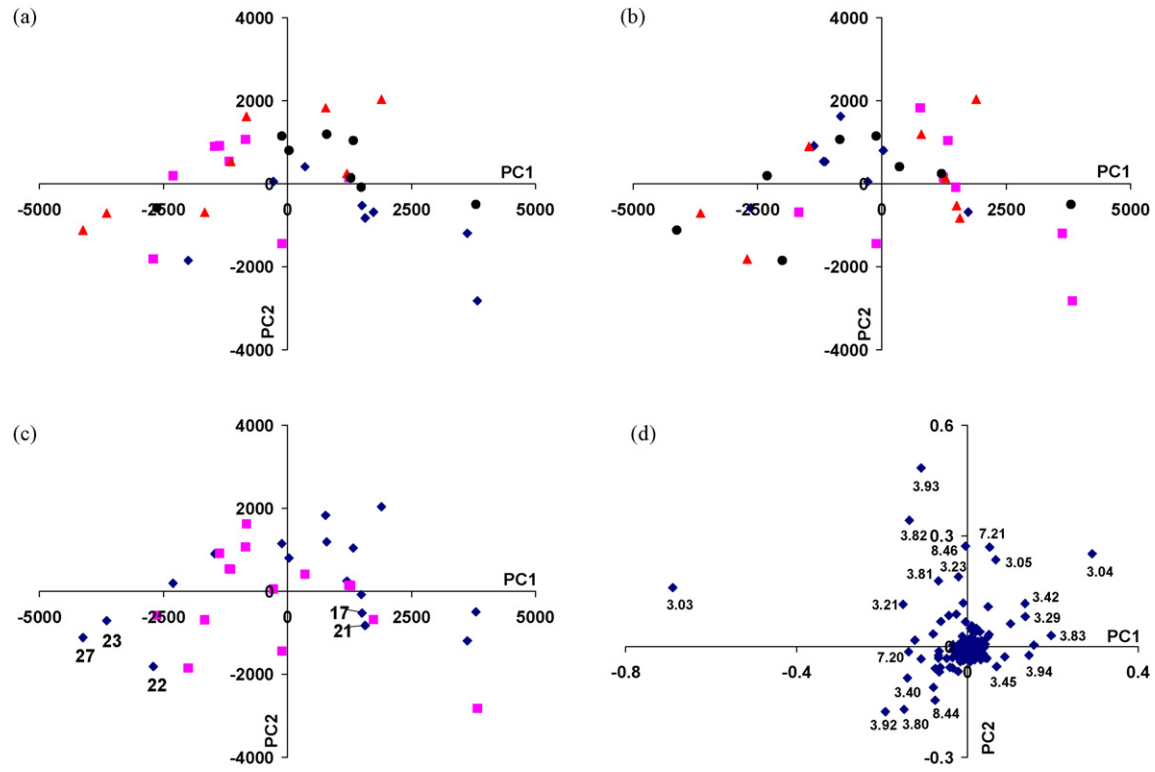

Fig. 4. Results from mean-centered PCA using the NMR spectra of mouse urine samples: (a) score plot of 32 untreated urine samples labeled according to different mice: blue diamonds: M053, pink squares: M054, orange triangles: M055, black circles: M056; (b) score plot labeled according to collection time: blue diamonds—first week, pink squares—second week, orange triangles—third week, black circles—fourth week; (c) score plot labeled according to original sample condition: blue diamonds—clean samples, pink squares—visible food or fecal particles; data points with number 17, 21, 22, 23 and 27 represent samples with volume more than 5 ml; (d) loading plot of the PCA result from 32 urine samples, with chemical shifts in ppm indicated.

lowing experiments were conducted. First, 12 "control" urine samples, which were free of any visible particles and had volumes below 5 ml, were selected from the 32 untreated samples for the following detailed PCA analysis: one sample (labeled as 9) from M053, two samples (18, 26) from M054, three samples (15, 19, 31) from M055 and six samples (8, 12, 16, 20, 28, 32) from M056. Fig. 5 shows the results of mean-centered PCA of the spectra obtained from the 12 "control" samples, plus 11 additional untreated samples which had larger collection volumes (also chosen from the 32 untreated samples), and all the contaminated samples. Urine samples soaked with folate-deficient food are clearly separated from the others in the score plot because of the introduction at high concentration of a number of metabolites. These metabolites that appear in the loading plot (Fig. 5b) include: glucose, creatine, creatinine, taurine and TMAO; PC1 (57% of the variance) expresses the main difference causing this distribution in Fig. 5a. It is also observed that urine samples mixed with fecal particles or turkey are clustered together with the 11 untreated samples and 12 "control" samples but with some separation along PC2 (PC2 explains 17%

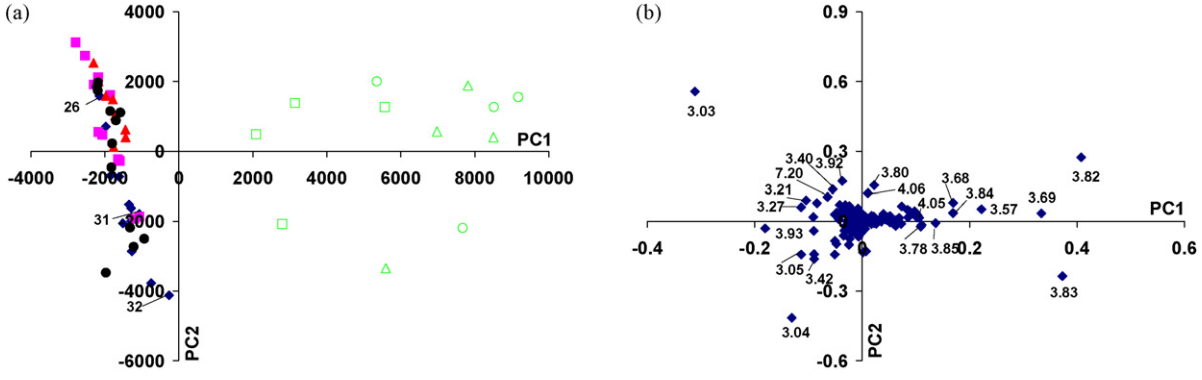

Fig. 5. Results from mean-centered PCA of the mouse urine NMR spectra: (a) score plot; solid blue diamonds—12 "control" samples, solid pink squares—11 untreated samples with larger volumes, solid orange triangles—fecal particle contaminated samples, solid black circles—turkey contaminated samples, green hollow symbols—folate-deficient food contaminated samples (circles: vortex preparation, squares: non-vortex preparation, triangles: incubation preparation); (b) the loading plot corresponding to (a).

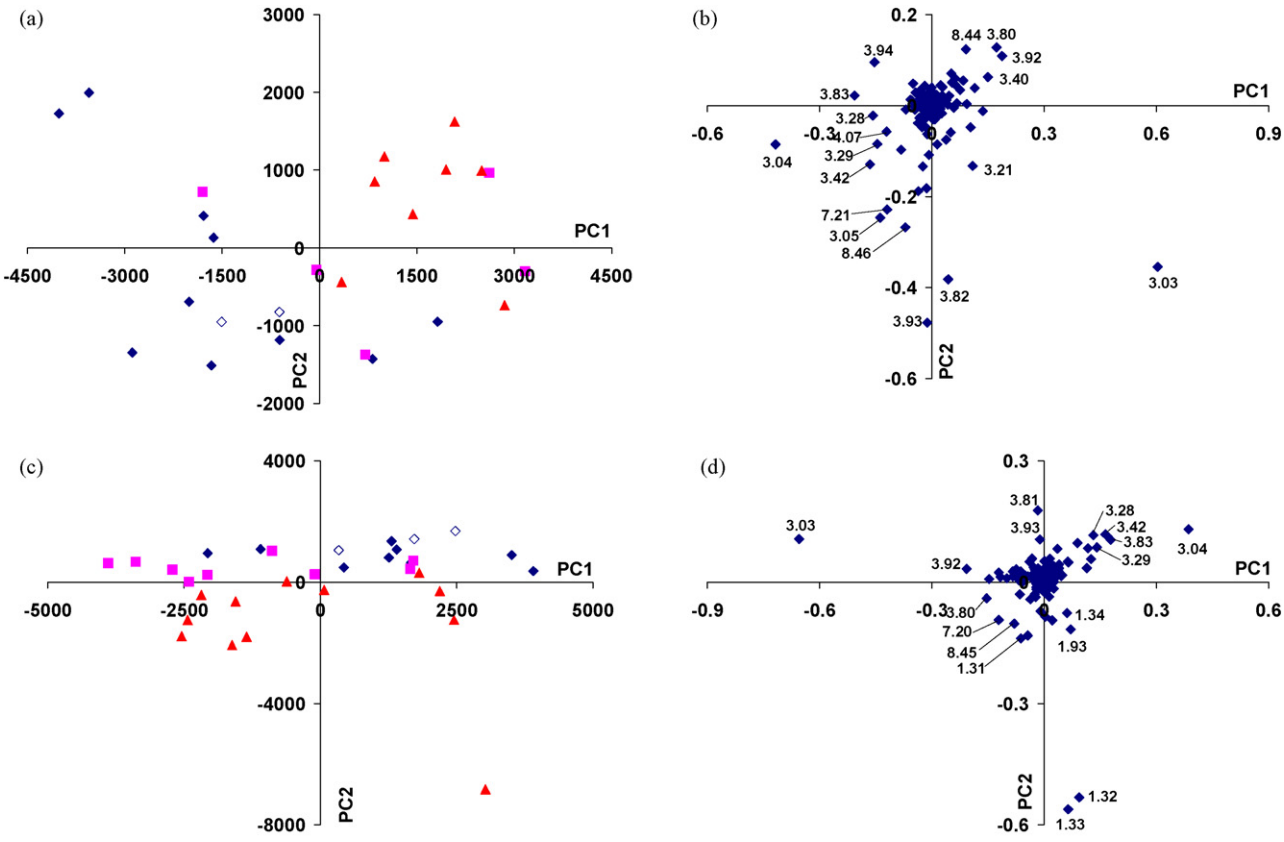

Fig. 6. Results from mean-centered PCA using the NMR spectra of mouse urine samples: (a) score plot for the fecal particle study; solid and hollow diamonds: 12 "control" samples (hollow diamonds represent two "control" samples used for mixing), pink squares: five additional untreated samples also used for fecal particle mixing, orange triangles: eight samples contaminated with fecal particles; (b) loading plot corresponding to (a); (c) score plot for the turkey study, solid and hollow diamonds: 12 "control" samples (hollow diamonds represent three "control" samples used for mixing), pink squares: nine additional untreated samples also used for turkey mixing, orange triangles: 12 samples contaminated with turkey; (d) the loading plot corresponding to (c).

of the variance). In addition, from the average (center of mass) of the points corresponding to the folate-deficient food/urine mixing experiments, it appears that samples prepared by vortex mixing (hollow green circles) are more different from the 12 "control" samples than the other two preparation methods, although the classification within these samples soaked with folate-deficient food is somewhat weak. These results likely occur because granular particles disperse faster than lumpy particles when mixed with liquid. The effect due to the incubation preparation can be decreased by storing samples at a lower temperature.

Samples for the folate-deficient food contamination study were removed from the PCA to focus on the effect of feces and turkey on urine, and these results are shown in Fig. 6. Spectra of 12 "control" samples (two of these were also used for mixing), five untreated samples (from the 32 untreated samples) for the fecal particle study, and samples contaminated by feces were selected for mean-centered PCA analysis (Fig. 6a). Mouse fecal particles have no significant effect on the mouse urine as indicated by the fact that the orange triangles (fecal particle contaminated samples) mix well with pink squares (five untreated samples), and there is no clear separation among contaminated samples due to different preparations as seen in the score plot. There is, however, a small difference between "control" samples and contaminated samples prepared by the different methods, as seen in Fig. 6a. Metabolites discussed above (glucose, creatine, creatinine, taurine and TMAO) are again principal species associated with the distribution in the score plot according to Fig. 6b. In Fig. 6c, 12 "control" samples, 9 additional untreated samples (used also for turkey mixing), and samples with turkey contamination, are all scattered along PC1 (which explains 44% of the variance), indicating that the effect of turkey contamination is very small. The variations between three categories of samples are expressed by PC2 (23% of the variance), although these differences are again too small to achieve good separation. The preparation method again appears to have no significant effect according to the score plot, which is most likely due to the fact that it is difficult for either turkey or fecal particles to dissolve in water. Compared to the loading plots above, the importance of lactate and acetate is increased, as large contributions of these metabolites to the loading plot appear in Fig. 6d. Also, changes in the glucose concentration appear to have a lesser effect as observed in the loading plots.

According to the PCA results above, turkey seems to provide an advantage in causing only a small amount of contamination in mouse urine samples. This conclusion is drawn from the similarity of the contaminated samples and "control" samples observed in the PCA results. However, some problems caused by feeding

mice turkey meat were noticed within the 24-h collection period. Mouse fecal particles became smaller, which made it harder to filter the urine, and in addition it was observed that the mice lost some weight during the urine collection periods. This is most likely due to a change in their environment from an enclosed cage with bedding and environmental enrichment to an open floored, singly housed cage where water and the different food were presented in a different apparatus. The mice did not experience a change in their activity or overall appearance during the whole of the collection period. The average weight of the mice at the end of the fourth week was almost the same as it was at the beginning of the study.

4. Conclusions

In an effort to provide a method to collect reliable mouse urine samples, changes in mouse urine mixed with folate-deficient food, turkey or fecal were evaluated by NMR spectroscopy and PCA. Particles mixed in mouse urine are most likely to be folate-deficient food, turkey slices, or fecal particles. Another problem observed during the collection period is that water may be spilled directly into urine, instead of being consumed by the mice. However, the PCA results show that after water peaks are carefully supressed in the NMR experiment and the 4.5–6.0 ppm region of each spectrum is removed prior to PCA, extra water causes no problems in terms of biasing the statistics. However, particles of folate-deficient food do appear to change the apparent metabolic profile of urine, while turkey or fecal particles have a negligible effect on the distribution of the sample data in the PCA score plots. Choosing turkey as a food source appears to meet the objective of achieving relatively uncontaminated urine. In order to maintain the healthy condition of the mice, this diet should be cycled. We have recently observed [27] that the turkey diet can have some effect on metabolites in the urea cycle and metabolism of amino groups, but such dietary changes are rapid from a metabolic perspective, at least for rats. Alternatively, perhaps a meat product with the texture comparable to real turkey, but the nutritional composition more suitable to mice, could be developed and used in future studies.

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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.jpba.2007.06.030.

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