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Metabonomics, dietary influences and cultural differences: a ^{1}H NMR-based study of urine samples obtained from healthy British and Swedish subjects

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Abstract

The aim of this study was to assess the feasibility and comparability of metabonomic data in clinical studies conducted in different countries without dietary restriction. A ^{1}H NMR-based metabonomic analysis was performed on urine samples obtained from two separate studies, both including male and female subjects. The first was on a group of healthy British subjects (n = 120), whilst the second was on healthy subjects from two European countries (Britain and Sweden, n = 30).

The subjects were asked to provide single, early morning urine samples collected on a single occasion.

The ^{1}H NMR spectra obtained for urine samples were visually inspected and analysed chemometrically using principal components analysis (PCA). These inspections highlighted outliers within the urine samples and displayed interesting differences, revealing characteristic dietary and cultural features between the subjects of both countries, such as high trimethylamine-*N*-oxide (TMAO)-excretion in the Swedish population and high taurine-excretion, due to the Atkins diet. This study suggests that the endogenous urinary profile is subject to distinct cultural and severe dietary influences and that great care needs to be taken in the interpretation of 'biomarkers of disease and response to drug therapy' for diagnostic purposes.
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1. Introduction

Metabonomics has found widespread application in the study of toxicological events in animal models [1–4]. Metabonomic studies can provide a quantitative description of low-molecular mass endogenous metabolites present in a biological sample such as urine, plasma or, although more invasive, tissue. The characteristic "metabolic fingerprint" profiles generated by high-field ^{1}H nuclear magnetic resonance (NMR) spectroscopy can be used to provide information, either visually and/or with suitable chemometric analysis such as principal components analysis (PCA), on gender, strain of animal, diurnal variation, response to toxic insult and disease [1–8]. The technique has, to date, been predominantly applied to studies in experimental animals. Potentially, metabonomics also has a role in monitoring the response to drug therapy. Although metabonomics will find increased application in the studies of both healthy [13] and diseased humans [9–12], one of the major obstacles in clinical investigations is

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the inherently greater variability in a human population compared to that seen in experimental animals. In human studies, environmental influences, such as diet, toxins including alcohol and nicotine, are much less readily controlled than when animal populations are investigated. Clearly, for reliable application of metabonomics in humans it is necessary to demonstrate that it would be possible to detect the effects of disease progression or drug therapy in subjects under clinical study conditions. Here, we describe two investigations on healthy subjects designed to evaluate the variability in metabonomic data and its utility for incorporation into clinical studies. As observed previously in the literature [15–18], metabonomic studies can be subject to dietary influences and cultural trends. These lifestyle effects were also highlighted and confirmed here by comparison of the urine samples from healthy subjects from two different northern European countries.

2. Experimental

2.1. Subjects

These studies were conducted in accordance with the ethical principles of Good Clinical Practice and the Declaration of Helsinki. The respective local Ethics Committees approved the protocol before commencement of the study, and all subjects gave written informed consent. Volunteers were recruited from the AstraZeneca Healthy Volunteer Panel. To be eligible to join the panel, subjects must be in good general health and not require regular medication other than oral contraception or HRT but may have minor conditions such as hayfever or migraine for which they require intermittent treatment. In addition, clinical chemistry and haematological parameters should be within acceptable limits.

2.2. Two independent evaluations were carried out

In the first evaluation, we investigated the variability of metabonomic parameters in 120 healthy human subjects. Inclusion/exclusion criteria simply required the volunteers to be between the age of 18 and 65 (mean ± S.D. = 39 ± 9 years) and to be a member of the AstraZeneca healthy volunteer panel. No subject restrictions were imposed.

The ^{1}H NMR analysis and PCA analysis were conducted without prior knowledge as to sex and age of the volunteers.

In the second evaluation, we aimed to determine cultural differences between British and Swedish volunteers. This group consisted of 20 healthy Swedish subjects (male and female) and 10 healthy British subjects (male and female) aged between 21 and 65 (mean ± S.D. = 53 ± 12 years). In addition to being eligible to be on the AstraZeneca healthy volunteer panel, the subjects completed a questionnaire, in which they confirmed their continued good health and that they were not taking any regular medication. Subjects were asked to fast overnight, abstain from drinking alcohol for 24 h and abstain from smoking for 24 h. The British subjects were asked to avoid cheese, fish and cherries (which are known to contribute to the urinary metabonomic profile) for 24 h before providing urine.

2.3. Food intake and medication

Excessive intake of alcohol (greater than 28 units per week) and drugs of abuse (e.g., methadone, benzodiazipines, cocaine, amphetamines, tetrahydro-cannabinal (THC), opiates and methamphetamines (ecstasy and barbiturates)) were prohibited.

Subjects were required to abstain from taking any medication (including over-the-counter remedies), where possible. However, subjects on regular medication or therapy (e.g., HRT and contraceptive pill) were granted continuous use/consumption. Subjects were generally allowed unrestricted food, but were asked to keep a basic dietary diary, where possible. This study design was supposed to mimic a situation in the clinic where people are typically on regular medication and receive a choice of foods.

2.4. Samples

First void urine samples were collected and were frozen at −20 °C on collection and stored frozen until analysis.

2.5. ^{1}H NMR Spectroscopy

Analysis of the urine samples was carried out by ^{1}H NMR spectroscopy on Bruker DRX500 spectrometer operating at 500.13 MHz ^{1}H resonance frequency. The NMR-probe used for that purpose was an SEI-microprobe and the samples were run in NMR-microtubes with a total volume of 250 μl.

For the urine samples an aliquot (3 ml) was freeze-dried prior to analysis and reconstituted into 500 μl of D_2O. A 200 μl aliquot of each urine concentrate was transferred into the NMR tubes, to which a further 50 μl of TSP (3-trimethylsilyl-$^2H_4$-propionic acid) in D_2O (1 mg/ml) was added. ^{1}H NMR spectra were acquired immediately after preparation of each individual sample. The D_2O provided a field frequency lock-solvent for the NMR spectrometer and the TSP served as an internal chemical shift reference ($\delta_{^1H}$ 0.0).

All spectra were recorded at 30 °C. Typically, ^{1}H NMR spectra were measured with 64 scans into 65536 data points over a spectral width of 9980.04 Hz, which resulted in an acquisition time of 3.28 s. A relaxation delay of 1.8 s additionally ensured T1-relaxation between successive scans. Solvent suppression of the residual water signals (a broad singlet at $\delta_{^1H}$ 4.8) was achieved via the Noesypresat pulse sequence (Bruker Biospin Ltd.) in which the residual water peak is irradiated during the relaxation delay and during the mixing time of 150 ms.

All ^{1}H NMR spectra were manually corrected for phase and baseline distortions within XWINNMR™ (version 2.6,

Bruker Biospin Ltd.). Spectra were referenced to TSP ($\delta_{^1H}$ 0.0) prior to data-reduction into 245 spectral integral regions corresponding to the chemical shift range of $\delta_{^1H}$ 0.2–10 utilising AMIX (version 2.7.5, Analysis of MIXtures, Bruker Biospin Ltd.). The region of $\delta_{^1H}$ 4.52–6.0 was set to zero to remove the effects of variations in the presaturation of the water resonance in all NMR spectra, and to alleviate cross-relaxation effects in the urea signal via solvent exchanging protons. Integration into bins (or buckets) across the spectral regions of 0.04 ppm was performed automatically in AMIX. Normalisation, i.e., making the total integrated intensity of each spectrum the same, compensated for the differences in overall concentration between individual urine samples. The resulting data matrix (peak integral values per bins per sample) was analysed by pattern recognition methods within SIMCA-P (version 8, UMETRICS AB, Box 7960, SE 90719 Umeå, Sweden) and/or visualised using "Spotfire" (Spotfire DecisionSite 6.2, version 6.2.0).

2.6. Statistical methods and software

Following the processing of the spectra by AMIX, data analysis was performed using various techniques including principal components analysis. The Spotfire program was used to visualise both the spectral data (reconstructed data-reduced spectra) and the output from SIMCA-P. Visualising the spectra in this way aided the identification of unusual spectra and individual peaks in the spectra. SIMCA-P was used to perform the principal components analysis. PCA was performed using centred scaling. Detailed accounts of pattern recognition methods can be found in the literature [14].

3. Results and discussion

In our previous study, we were concerned with the variability of metabonomic data in a group of healthy male volunteers housed within a clinical pharmacology unit under strict dietary and lifestyle restrictions [13]. In the current study, we attempted to investigate the variability of a healthy control human population of both sexes without strict exclusion criteria. The aim of the study was to examine variability in a typical clinical study population and assess the reliability of methods for detecting outliers by metabonomics. We also hoped to detect dietary patterns and confirm possible cultural trends within volunteers from two northern European countries.

Sound knowledge of normal variability and dietary metabolites is a prerequisite for the correct interpretation of metabonomics data, in order not to confuse the results with biomarkers of disease or toxicity.

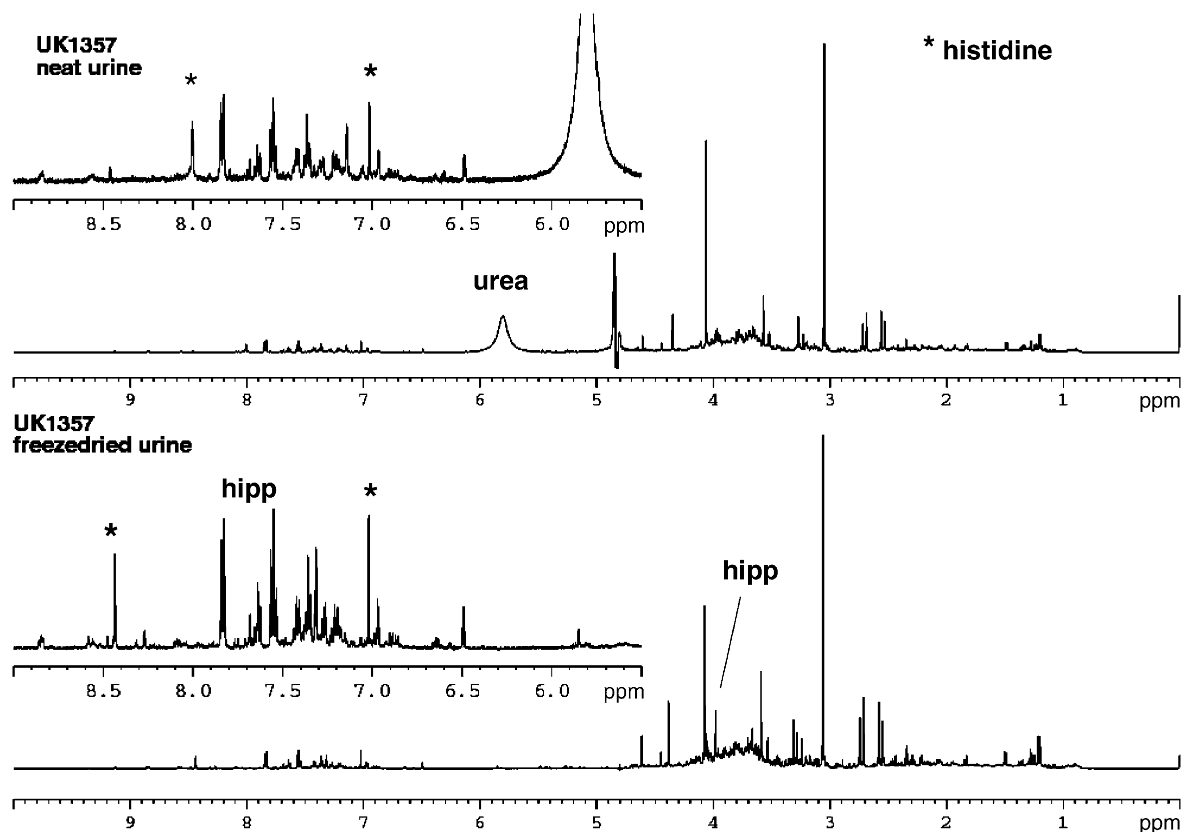

Fig. 1. The effect of freeze-drying: ^{1}H NMR spectra of freeze-dried and neat urine from the same volunteer.

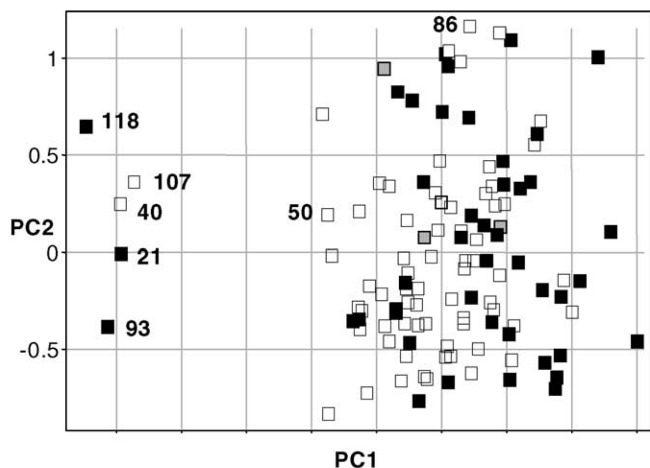

Fig. 2. PCA scores plot from the urines from 120 British healthy volunteers, displaying distinct outliers (urines 118, 107, 40, 21 and 93). Key: black = male; white = female; and grey = sex not disclosed.

3.1. Demography

For the first evaluation, a group of 120 British human volunteers (average age ± S.D. = 39 ± 9 years) was recruited to assess the inter-subject variability in a given healthy population without food restriction in order to assess the reliability of PCA as a first screen for outliers in clinical studies. This study was initially conducted ‘blind’, i.e., no information as to the sex and diet were provided, and outliers were sought by visual inspection of the ^{1}H NMR (raw) data and PCA.

A further group of thirty healthy male and female subjects was recruited for the trial for evaluation 2, consisting of 10 British and 20 Swedish volunteers with an average age of 53 ± 12 years (mean ± S.D.), in order to highlight and confirm any cultural differences.

3.2. Urine

Human urines are relatively dilute and with the probe design used here (185 μl sample volume), required relatively long spectral acquisition times. As in our previous study, the urine samples were freeze-dried and re-dissolved in a smaller volume of D_2O in order to concentrate them [13]. Samples were analysed immediately following reconstitution in D_2O, unless stated otherwise.

For the multivariate statistical analysis, each urine spectrum was first data reduced within the AMIX software and then reconstructed within the Spotfire data visualisation package. Visual inspection and investigations within Spotfire highlighted unusual spectra and differences between the urine samples and, generally, these observations were confirmed by PCA analysis.

4. Evaluation 1: variability within a group of 120 healthy British volunteers

4.1. Investigation of effect of freeze-drying

A typical urine spectrum for one of the volunteers, prior and post freeze-drying, is displayed in Fig. 1. The figure

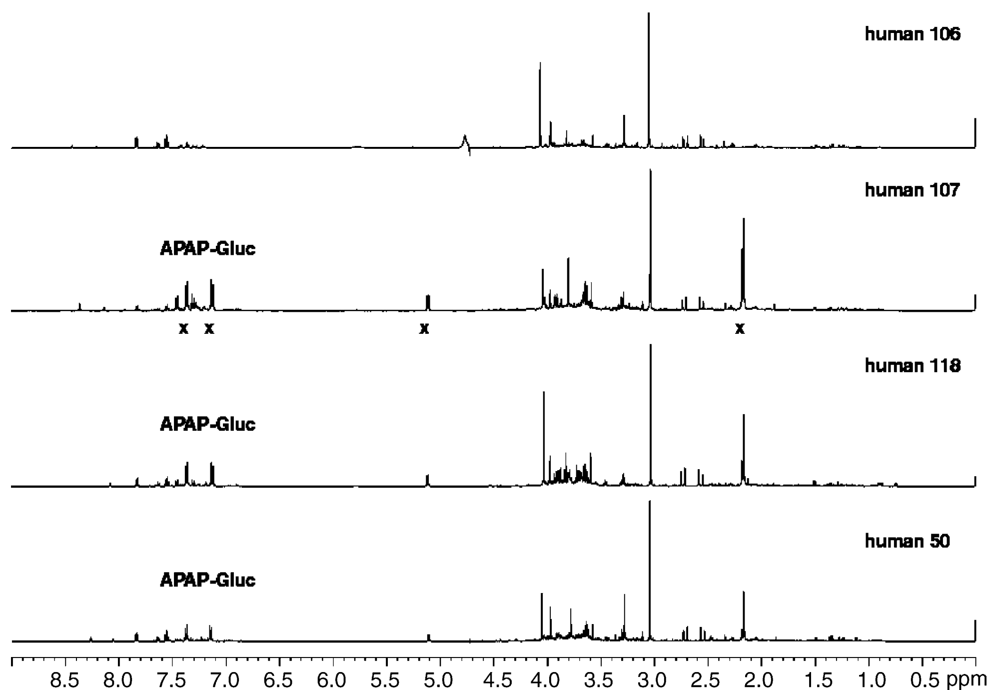

Fig. 3. ^{1}H NMR spectra of urines from outliers (urines 107 and 118) displaying paracetamol glucuronide signals (x = diagnostic APAP-gluc signals). Urine no. 50 contained the paracetamol glucuronide signals but was not identified as outlier by PCA. Urine sample no. 106 serves as control.

shows that the urine spectra are compatible, however, changes following freeze-drying and reconstitution in D_2O included the shift of the histidine/carnosine resonance to higher frequency (from $\delta_{^1H}$ 8.01 to 8.42), the exchange of the urea protons with the deuterium of the solvent (D_2O) and the collapse of the glycine doublet from hippurate ($\delta_{^1H}$ 3.97) into a singlet. Despite the restriction on alcohol, the NMR spectrum of the neat urine from one volunteer was found to contain unusually high levels of ethanol (EtOH), the signals of which had disappeared as a consequence of freeze-drying (data not shown). However, since all the samples were freeze-dried to speed up analysis by NMR spectroscopy, information regarding volatiles, such as EtOH, was lost as a consequence of sample preparation, throughout.

A comparison between visual inspection of the ^{1}H NMR spectra, obtained from the freeze-dried urines, and the corresponding data interrogated independently via PCA was carried out. Distinct outliers could be observed with both approaches, although visual inspection highlighted a larger number of unusual spectra.

The volunteer panel consisted of a mixture of sexes, which were 'colour' coded for comparison in the PCA-scores plot (Fig. 2). This information was initially withheld, as not to bias the outcome. Neither visual inspection of the ^{1}H NMR spectra nor PCA analysis could differentiate between the sexes, even when this information was subsequently included in the data analysis. The lack of a clear separation was, however, not surprising, in view of the known degree of variation within a human population.

The data set examined by PCA analysis revealed five distinct outliers as shown in Fig. 2. Visual inspection of the corresponding ^{1}H NMR spectra clearly showed metabolites of paracetamol (paracetamol glucuronide) in urine samples 107 and 118, resonances of unknown origin (urine sample 40, data not shown) and two samples (no. 21 and 93) displaying loss of a signal in the ^{1}H NMR spectrum. These two samples were analysed following storage at room temperature overnight, which caused the methylene-signal of creatinine (a singlet at $\delta_{^1H}$ 4.05) to be exchanged with the deuterium of the solvent. The proposed mechanism is based on keto-enol-tautomerism, resulting in the disappearance of the ^{1}H-signal as a consequence of 'sample storage' (data not shown).

However, more importantly, urine sample 50 was not identified as an outlier by PCA despite the presence of paracetamol metabolite signals (paracetamol glucuronide, Fig. 3).

It was, hence, of interest whether urine sample 50 could be resolved by PCA. This was investigated by conducting the chemometric analysis after omitting the 5 original outliers and re-subjecting the residual data set to PCA analysis. The resulting scores plot of PC1 versus PC2 did not reveal sample 50 or indeed any other sample as an outlier (Fig. 4a), whereas PC1 versus PC3 flagged up a distinct outlier (Fig. 4b). However, this outlier was not sample 50 but represented urine sample 86, a sample high in TMAO, presumably due to a fish-diet prior to collection [15–17].

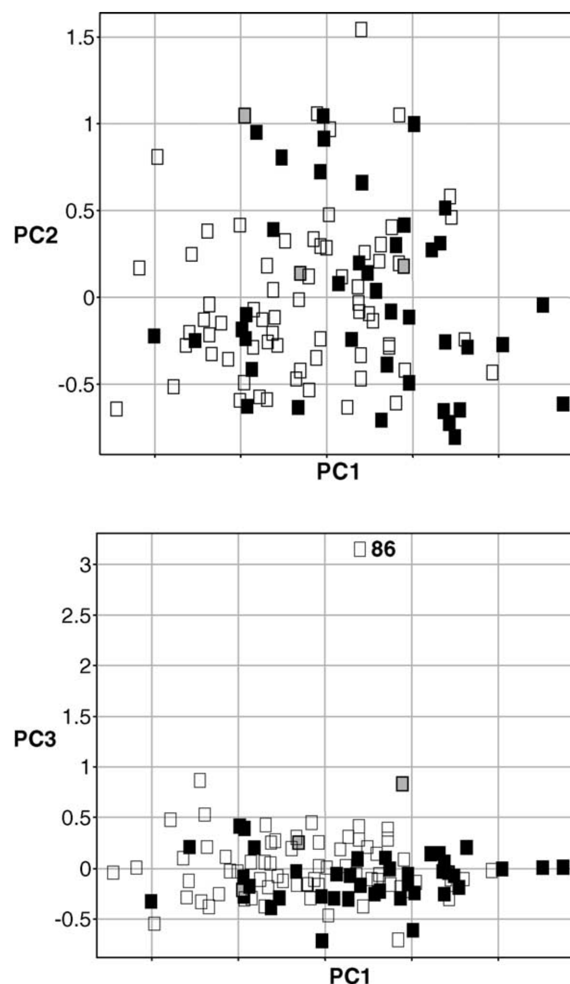

Fig. 4. PCA scores plot excluding the outlying urines (see Fig. 2). The residual data set was re-subjected to PCA analysis, showing (a) the scores plot of PC1 vs. PC2 and (b) displaying the scores plot of PC1 vs. PC3. Key: black = male; white = female; and grey = sex not disclosed.

Visual inspection of all the urine spectra revealed several further unusual spectra in addition to the ones identified by PCA. These spectra were characterised by elevated concentrations of TMAO (fish-diets), the presence of mannitol (presumably from chewing gum) or of some unknown and, hence, unassigned resonances.

This study has highlighted the importance of visual inspection of the raw ^{1}H NMR data, as all outliers could not be reliably identified by PCA analysis as a preliminary screen for clinical studies. However, since this evaluation was conducted to mimic a situation typically encountered in a clinical study/hospital environment, where dietary restrictions, etc., can be limited, it suggests that such data inspection is important for the integrity of studies.

The effect of sample storage and signal stability is also of importance, especially when samples are queued for analysis. The loss of a dominant signal due to chemical exchange with the deuterated solvent can have a large influence on the resulting PCA scores-plot. Again, interrogation of the raw data

is required as to explain the finding in order not to discriminate against ‘outliers/patients/volunteers’ unjustly. Clearly, chemical exchange was exacerbated here by the analytical protocol forced on us by limited probe capacity and need to use freeze-dried samples redissolved in D_2O. However, even where freeze-drying is not used some D_2O is generally added to samples as a field frequency lock and a variable amount of exchange is therefore likely to occur.

5. Evaluation 2: investigation of cultural differences

As with evaluation 1, for the multivariate statistical analysis, each urine spectrum was first data reduced within the AMIX software and then reconstructed within the Spotfire data visualisation package, as well as interrogated by PCA.

The PCA generated scores plot enabled some distinction to be made between the British and Swedish volunteers (Fig. 5a). The metabolites responsible for distinguishing between the populations are indicated in the loading plot (Fig. 5b) as hippurate, TMAO and creatinine. In addition, taurine was unusually high in one of the British subjects (urine sample 6). Visual inspection and comparisons within Spotfire confirmed these differences. However, as with evaluation 1, some of the urines containing signals from mannitol were not identified by PCA, but could be observed by visual inspection of the raw data (^{1}H NMR spectra).

Nevertheless, examination within Spotfire highlighted some clear cultural trends. Hippurate excretion appeared to be higher in the Swedish population (especially urine samples 15 and 22), while the British population excreted higher concentrations of creatinine (although urine sample 23 from the Swedish group also contained high creatinine).

There was also a clear distinction in TMAO excretion levels, especially for urine samples 12, 13, 26 and 29 from the Swedish group. The high TMAO concentrations have previously been attributed to fish-diets [15–17], possibly not a surprising observation with the Swedish subjects. The corresponding ^{1}H NMR spectra are shown in Fig. 6.

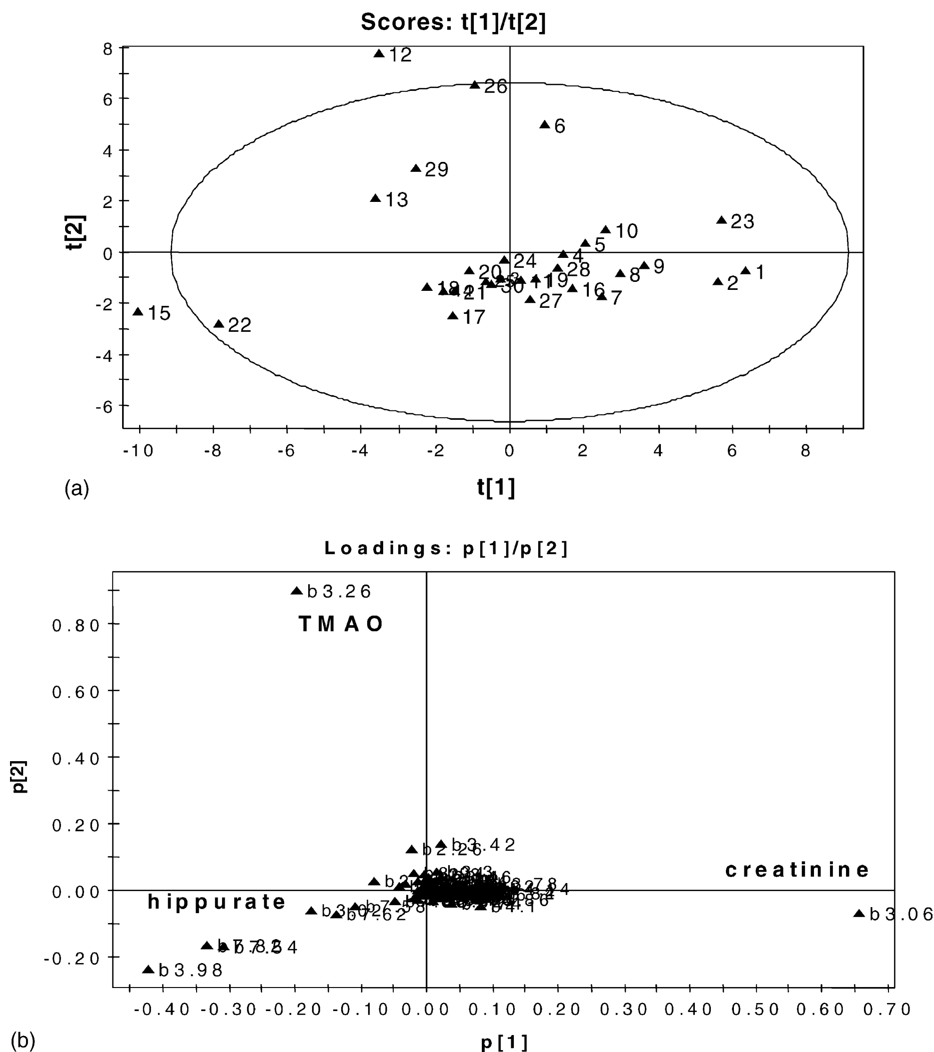

Fig. 5. Cultural differences highlighted by PCA of urines from Swedish and British volunteers (a) the PCA scores plot (PC1 vs. PC2) and (b) the corresponding loadings plot. Key: urine samples no. 1–10 are from the British subjects, no. 11–30 from the Swedish subjects.

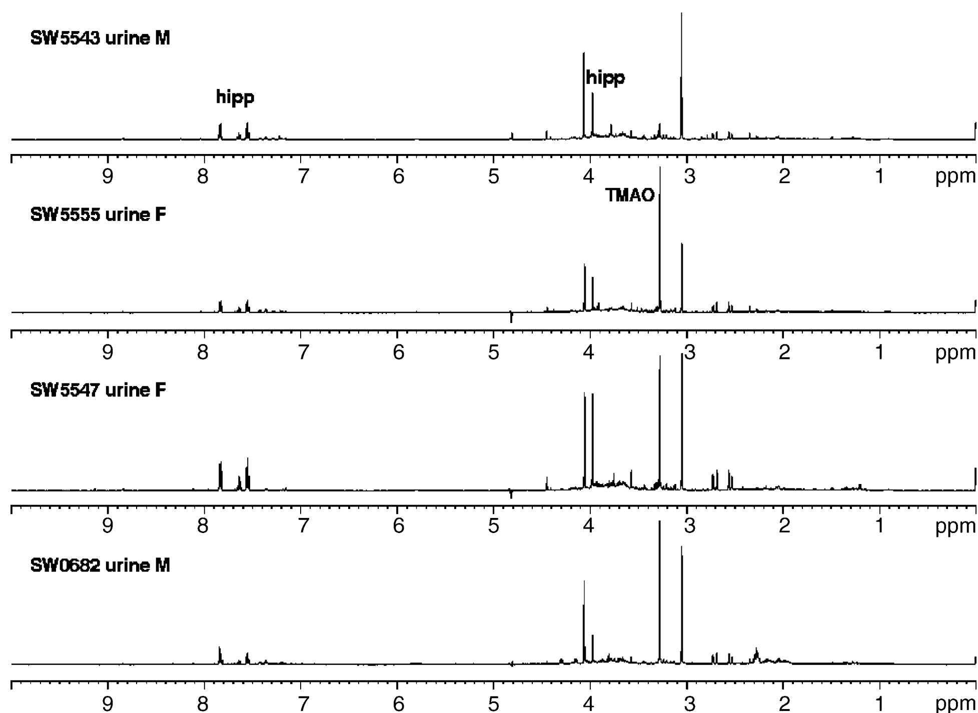

Fig. 6. ^{1}H NMR spectra of urine samples from representative Swedish subjects, displaying the characteristic high excretion of TMAO in some of the subjects.

The urine (no. 6) from the female British volunteer showed unusually high levels of taurine (Fig. 7). This particular urinary metabolite has been reported in the literature as a possible indicator of liver toxicity [19]. However, the volunteer in question has been regular blood donor and volunteer and has generally displayed normal liver function. At the time of the study, the volunteer followed the Atkins diet, a diet rich in meat, which in turn can contain high concentrations of taurine [20–23]. A repeat urine sample, approximately one year later, clearly showed that the taurine was not elevated (Fig. 8). This sample was acquired without freeze-drying, hence, exposing some alcohol consumption (EtOH peaks). The volunteer had

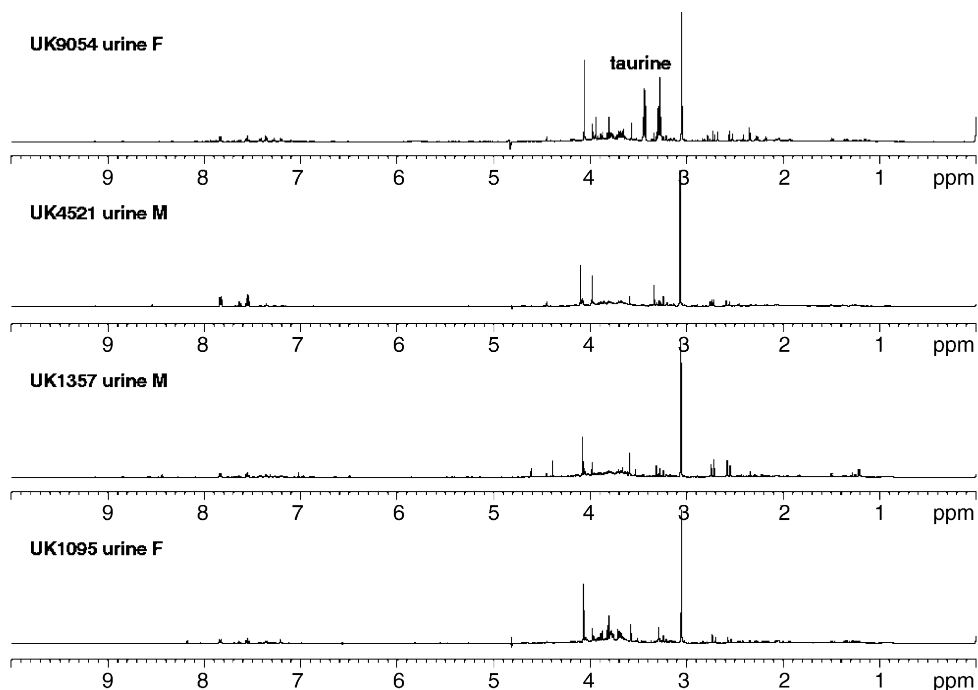

Fig. 7. ^{1}H NMR spectra of the urines from representative British subjects. Urine sample no. 6 from female volunteer 9054 displaying unusually high taurine.

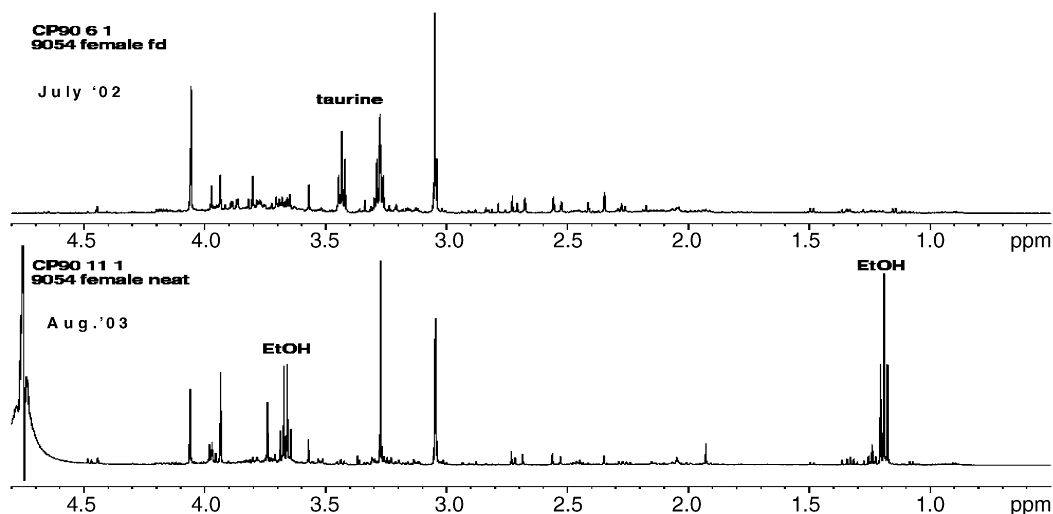

Fig. 8. ^{1}H NMR urine spectrum from British female volunteer 9054 displaying high concentrations of taurine due to the Atkins diet. Shown are the spectra during the diet in July 2002 (freeze-dried) and approximately one year later (neat).

clearly also consumed some fish prior to urine collection, as indicated by the presence of the large TMAO peak ($\delta_{^1H}$ 3.27). In addition, the sample also contained a smaller ethanol related signal, probably corresponding to ethyl glucoside. This is a component of rice wine (which is used in cooking) and Saki and is associated with consumption of oriental food and beverages [24]. It should be pointed out that whilst the second sample was collected outside the main part of this study, it again reveals dietary and lifestyle influences and cultural trends.

The dietary trends and lifestyle influences can be a potential issue particularly for longitudinal studies, where lifestyle restrictions are outside the control of the triallists.

These findings reinforce the importance of taking into account the effects of dietary influences on sample composition in "omic" studies, if meaningful information is to be extracted.

As pointed out in our previous publication, for urine and plasma samples, a standard diet and lifestyle restrictions should be considered in clinical studies [13], if possible.

6. Conclusions

The results of evaluation 1 have shown that PCA as a quick screen to expose outliers as part of a clinical study has potential, although additional visual inspection of the original raw data (^{1}H NMR spectra) is required. The urines are clearly prone to variability, which is due to dietary and lifestyle influences.

The consumption of medication and ethanol was detected in some cases, even though concomitant medication and excessive alcohol consumption were excluded by the protocols. Hence, the application of metabonomics appears to prove useful in the identification and subsequent exclusion of non-compliant individuals from the trial. However, we have shown that despite PCA interrogation of the NMR data, some outliers can remain undetected and visual inspection of the raw data is highly recommended.

Separation on the basis of gender could not be achieved in this study, however, based on the known degree of variability within the data set, this was not surprising. Sample storage and lability of some signals in the spectrum also has to be considered, especially when samples are stored for a long time, such as in a queuing system in an automated set-up.

Interestingly, some cultural differences could be observed and confirmed between Swedish and British volunteers in evaluation 2. The differences were mainly based on the dietary preferences of the Swedish subjects (fish-diet and TMAO-excretion). A more dramatic effect of diet was evident in one British volunteer having followed the Atkins diet at the time of the study, showing unusually high levels of taurine, a urinary metabolite commonly regarded in the literature as a biomarker for hepatotoxicity.

In summary, we have demonstrated the degree of variability, which might be seen in metabonomic data derived from patients and healthy volunteer studies. It is highly likely that dietary and other cultural influences contribute to the variability in metabonomics parameters. To allow successful interpretation of metabonomic data, triallists should either impose dietary and lifestyle restrictions or if restrictions cannot be imposed in such clinical trials, a dietary record should be considered. The period over which diet should be controlled is still unclear.

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