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Biofluid ^{1}H NMR-based metabonomic techniques in nutrition research — metabolic effects of dietary isoflavones in humans

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Abstract

A metabonomic approach to nutrition research may provide an insight into in vivo mechanisms of action following nutritional intervention. This approach was applied to investigate changes in the ^{1}H NMR spectral profile of urine collected from controlled dietary intervention studies conducted in premenopausal women before and following soy or miso consumption. The aim of the study was to identify the biochemical effects of a diet rich in soy isoflavones, phytochemicals which are receiving significant attention because of their potential importance to human health and wide bioactivity in vitro. By applying various chemometric techniques to the data the biochemical effects of conjugated and unconjugated isoflavones were determined. The biochemical changes observed suggest that soy isoflavone ingestion had significant effects on several metabolic pathways associated with osmolyte fluctuation and energy metabolism. These biochemical changes were more significant following ingestion of the unconjugated soy isoflavone (miso) diet suggesting that the chemical composition of the isoflavones present in soy-based foods may have an effect on their biological efficacy in vivo. This study describes a novel application for ^{1}H NMR analysis by determining subtle differences in biochemical profiles following dietary intervention and providing further insight into the mechanisms of action of phytochemicals in vivo.

1. Introduction

Interest in the relative importance of phytochemicals to human health has increased dramatically over the last decade with particular interest in relation to the class of compounds known as the phytoestrogens, which embody several groups of compounds including isoflavones, lignans, coumestans and prenyl flavonoids [1]. These compounds exert a wide range of hormonal and nonhormonal activities in animals or in vitro, and these suggest plausible mechanisms for potential physiological effects of diets rich in these compounds in humans [2]. In addition, experimental and epidemiological data are available to support the concept that isoflavone-rich diets exert physiological effects and preliminary human studies suggest a potential role in hormone-dependent diseases [1].

The biological actions of isoflavones are complex, and their ultimate cellular actions are determined by many factors including the relative levels of oestrogen receptor (ER) α and β, the diverse mixture of coactivators and corepressors present in any given cell type, and the nature of the response elements with which the receptors interact on the oestrogen-regulated genes [3]. It is therefore not surprising that the resulting effects observed from available in vitro and in vivo experiments are inconsistent since the biological effects vary

Abbreviations: AMIX program, analysis of mixtures; d, chemical shift; COSY, correlation spectroscopy; DMA, dimethylamine; DMG, dimethylglycine; ER, oestrogen receptor; FID, free induction decay; FMO3, flavin-containing monooxygenase; 3FT, Fourier transform; OSC, orthogonal signal correction; 2-OG, 2-oxoglutarate; ^{1}H NMR, proton nuclear magnetic resonance; PABA, *para* amino benzoic acid; PCA, principal component analysis; TCA, tricarboxylic acid cycle; TMA, trimethyamines; TMAO, trimethylamine-N-oxide; TSP, 3(trimethylsilyl)propionic-(2,2,3,3-d_4) acid.

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depending on the phytoestrogen compound studied, cell line used, species and tissue under examination. Although the reported oestrogenic potency of isoflavones is weak, 100–1000 times less compared with 17-β-oestradiol, their biological potential cannot be ignored, as typical circulating levels of isoflavones can exceed endogenous estradiol concentrations by 10,000-fold following consumption of a diet containing soy foods [4]. The isoflavone genistein is a potent agonist for ERβ, and the divergent transcriptional activities of oestrogens and isoflavones result not only from their different binding affinities but also from differences in their ability to recruit coregulators and trigger transcriptional functions of ERα and ERβ [5]. Numerous other biological effects independent of the ER (e.g., antioxidant capacity, antiproliferative and antiangiogenic effects) have been ascribed to phytoestrogens and many of these mechanisms are common to other plant phenolics [1,2].

In order to identify the in vivo biochemical effects of dietary phytoestrogens in humans, a metabonomic approach based on ^{1}H NMR spectroscopy of human urine was applied to investigate the biochemical effects of a diet rich in phytoestrogens in premenopausal women. Metabonomics is defined as "The quantitative measurement of time-related multiparametric metabolic responses of an intact living system to pathophysiological stimuli or genetic modification and thus provides a systems approach to understanding metabolic variation in complex multicellular organisms. Successful applications of this metabonomic approach in toxicity screening, drug metabolism and functional genomics have been documented, but these studies have mainly focused on the analysis of data from animals rather than humans [6], due to the greater control of condition in such studies to reduce extrinsic variability. The variability in the composition of urine is significant as the profile is affected by numerous environmental factors, for example, diurnal variation and estrus cycle [7]. In addition, with human data intersubject variation in metabolism is generally significantly greater than that observed in laboratory animal models because of their greater diversity in genetic and environmental factors. One method of minimising some of these environmental conditions in humans is by conducting carefully controlled dietary intervention studies, where each subject consumes a defined diet and participates in both a control and test intervention study period. Following dietary intervention, sophisticated techniques, like metabonomics, are required to determine accurately the subtle metabolic effects related to dietary change. In the current study, the availability of 24-h urine samples from such a controlled intervention study enabled the evaluation and determination of the suitability of this in vivo approach for the assessment of biochemical effects in vivo following a nutrition intervention in a human population.

Although in the past metabonomic techniques using ^{1}H NMR spectroscopy have previously been applied in toxicological studies [6], their application to human data is to date limited. In the area of nutrition research, a metabonomic approach for urine analysis has never previously been reported, although such an approach is complementary to the application of other proteomic and genomic tools to further develop functional biomarkers to determine subtle differences in biochemical profiles following dietary intervention.

2. Experimental protocol and sample collection

Complete 24-h urine samples were obtained as previously described by Cassidy et al. [8,9]. Healthy nonvegetarian women (21–29 years of age) were enrolled in the study. All women had regular, ovulatory menstrual cycles and had taken no medication for $\geq$6 months before starting the study. For two complete menstrual cycles, diets and physical activities were closely monitored and controlled. Basal metabolic rate was estimated from body weight, and the energy intake necessary to maintain a constant body weight throughout the study was calculated, assuming a ratio of total energy intake to basal metabolic rate of 1.4:1.0, to ensure that any observed changes in energy metabolism were not due to changes in the dietary energy content.

The first complete menstrual cycle served as a control period during which time each subject consumed a constant daily diet of non-soy-containing foods provided by a metabolic kitchen [1,2]. During the second month and starting on the first day of menses, appropriate modifications were made to the basal diet to maintain the amounts of micronutrients and nonstarch polysaccharides with the addition of either 60 g/day (dry weight) textured vegetable protein/day (n=6) or 50 g miso/day (n=3) to meals. These two foods were chosen as they contain isoflavones in different chemical forms conjugated (glucosides) or unconjugated (aglycones), respectively. All meals were prepared in advance, accurately weighted and deep-frozen until required. All frozen and canned foods were of the same batch to minimize interbatch variability, and bread that contained no soy flour was specially prepared for the study. The same batch of soy protein was used throughout the study period.

During each diet period 24-h urine samples were obtained at 3-day intervals. Urine volumes were recorded and aliquots taken and stored at −20°C. As an exogenous marker to monitor compliance with the dietary intervention, and to ensure completeness of the 24-h urine collections, the volunteers were given PABA check tablets (Laboratory for Applied Biology, London) [10] on the 24-h urine collection days. These urine samples allowed a comparative analysis of the biological effects of both conjugated (soy-texturised vegetable protein—TVP) and unconjugated isoflavones (miso).

3. Acquisition of NMR spectra and data analysis

^{1}H NMR spectra were acquired on a Bruker Avance DRX 600 spectrometer operating at 600.13 MHz at 303 K. Samples were analysed in 5-mm-od NMR tubes using a triple axis inverse (TXI) gradient probe.

4. Acquisition of 1D ^{1}H NMR spectra of urine

Urine samples were thawed and a 500-μl aliquot of urine and 200 μl 0.2 M sodium phosphate buffer pH 7.3 [containing 0.5% (w/v) NaN_3] were combined in a 1.5-ml polypropylene microcentrifuge tube. The samples were left to stand for 10 min and then centrifuged at 13,000 rpm for 10 min. An aliquot of 600 μl of supernatant was then added to 50 μl of 1 mM 3-(trimethylsilyl) propionic-(2,2,3,3,-d_4)-acid sodium salt (TSP) in D_2O. Standard 1D spectra were acquired using a standard pulse sequence for water peak suppression [i.e., RD−90°−t_1−90°−t_m−90°−acquired free induction decay (FID)] [11], where a recycle delay (RD) of 2 s during which the water resonance was selectively irradiated and t_1 corresponds to a fixed interval of 10 μs was used. The water resonance was irradiated for a second time during the mixing time t_m (100 ms). A total of 128 transients were acquired into 32 k data points using a spectral width of 14 ppm and an acquisition time of 1.84 s. Prior to Fourier transformation (FT), an exponential line broadening function of 0.3 Hz was applied to the FID.

5. Acquisition of urine 2D-^{1}H–^{1}H NMR spectra

Two-dimensional NMR spectra were acquired for selected samples including gradient 2D homonuclear ^{1}H shift correlated spectroscopy (COSY) using a presaturation pulse sequence (applying gradients instead of phase cycling) and 2D ^{1}H J resolved with presaturation [11]. Spectra were analysed visually and spectral subtraction was applied for the analysis of COSY data to aid the identification of metabolite resonances and structural elucidation.

A total of 64 transients were acquired into 2 k data points. A total of 256 increments were measured in F1 using a spectral width of 10 ppm and an acquisition time of 0.17 and 0.14 s was used for the COSY and J resolved experiments, respectively. The data were multiplied by a shifted Qsine-bell function prior to FT. Spectra were corrected for phase and calibrated to TSP (δ 0.0).

6. Data reduction

Standard ^{1}H NMR spectra of urine were automatically data reduced to 256 integral segments using the AMIX program (Analysis of MIXtures software package, version 2.5, Bruker Analtische Messtechnik, Karlsruhe, Germany), and the residual water region and urea were removed, to remove artefactual interference with the chemometric analysis. The resulting table of spectral intensity information was then exported to Microsoft Excel version 97 SR-2, where each spectral intensity data set was normalised to the total sum of the spectrum in order to reduce the effect of difference in the concentrations of the individual urine samples. The data were then exported into SIMCA-P (version 8.0 Umetrics AB package, Umes, Sweden). All subsequent analysis used centered data where the average value of each variable was calculated and subtracted from the data, such as is typically used where the measurement units for variables are in the same scale of magnitude, e.g., spectroscopic values [12,13].

7. Principal component analysis of the ^{1}H spectral data

Principal component analysis (PCA) is a multivariate projection method useful in handling large complex data sets [12–15]. It is usually applied in the initial stages of an investigation to present an overview of the information contained in a data set in a statistical manner. PCA creates a condensed summary of the data, which can be analysed graphically by means of two types of plots, the scores plot and the loadings plot. The scores plot is a summary of the relationship between the observations (i.e., spectra) and can be used to establish any significant pattern in the data; the loadings plot is a similar summary of the variables (i.e., the spectral integrals). The loadings can be viewed as a means to interpret the pattern seen in the scores plot, as the two plots are complementary. Thus PCA can facilitate the simultaneous comparison of a large number of complex objects such as biofluid spectra and provide information on biochemical (metabolite) changes with relation to physiological variation [12–15]. PCA was applied to the centred spectral data in order to explore any clustering behavior of the samples based on intrinsic biochemical similarities or differences, and thus determine whether intersample and intervariable relationships existed between the spectral profile of urine samples collected before and after soy intervention.

Models were constructed using all samples; outlier diagnostic plots based on the Hotelling's test (a multivariate approximation of the Student's t test) were examined in order to identify samples that mapped separately from the main data set grouping (i.e., outside the 95% confidence area based on their biochemical profiles). These spectra were analysed for artifacts and eliminated where necessary and PCA was repeated on the remaining samples. The scores plot of various PCs was examined for spectral clustering based on both diet and intersubject variation.

8. Data filtration (application of spectral filters)

In order to optimize the clustering of samples based purely on dietary variation rather than intersubject differences, a spectral filter orthogonal signal correction (OSC) was applied to the data prior to reanalysis by PCA. OSC can generate improved models particularly where large amounts of variance not related to the property of interest are described in the data. Information based on class (**Y** matrix — consisting of a single variable relating to dietary intervention) is used, to eliminate extraneous variance from the **X** data matrix that is unrelated to class (**Y** matrix) (i.e., components or latent variables orthogonal to the response calibrated against) producing new weight-

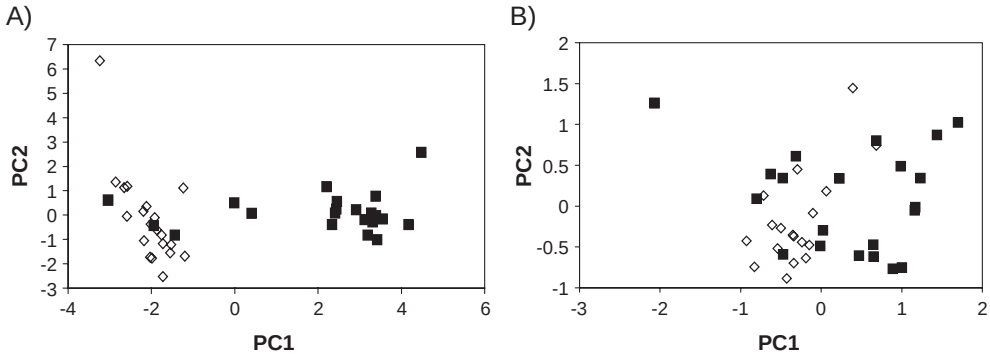

Fig. 1. The PC1 and PC2 scores plot based on mean centred descriptors derived from urinary ^{1}H NMR spectra illustrates the separation between spectra collected from subjects pre- (■) and post-miso (◇). (A) PCA scores plot based on MC descriptors. (B) PCA scores plot based on MC descriptors after elimination of the dominating variable (3.26) relating to trimethylamine-*N*-oxide (TMAO).

ing coefficients on the variables. Common sources of extraneous variation in such studies include, but are not limited to, age, genetic background, body mass, level of activity, state of health, etc. The orthogonality between the calculated components describing the variation in the spectra (**X**) and the response vector or variable (**Y**) ensures that only unwanted variation is removed. Having removed the residual matrix corresponding to extraneous variation, the OSC models will have fewer components making interpretation easier and enhancing the predictive ability of the model [16]. Furthermore, analysis of the removed orthogonal variables enables the identification and interpretation of the source of interfering variation within the data, which in itself may contain relevant information of biological importance.

In order to establish the robustness of the data after removing one orthogonal component and to ensure that data were not overfitted, it is essential to build training and test models with which to determine the predictability of the OSC model. In this study the original spectral data (**X** matrix) were separated into two dummy variables (**Y**) relating to class, i.e., class 0 (pre-TVP) and class 1 (post-TVP), before the OSC spectral filter was applied and chemometric analysis (PCA) carried out testing the robustness on the method.

Table 1
Summary of the key variables significant to the separation: between pre- and post-soy intervention phase (subjects 2–6) and between pre- and post-miso intervention phase

Loadings	Metabolite	Subject (SOY)					
		2	3	4	5	6	Miso
1.34, 4.1	Lactate	–	–	–	–	–	↓
2.22, 2.04	Unidentified[a]	↑	↑	↑	↑	↑	↑
2.66, 2.54	Citrate	↑	↑	↑	↑	–	↓
2.58	Methylamine	↑	↑	↑	↑	–	↑
2.7, 2.74	Dimethylamine	↑	↑	↑	↑	↑	↑
3.06, 3.94	Creatine	↑	↑	↑	–	↑	↑
3.1, 4.06	Creatinine	↓	↓	↓	↓	↓	↓
3.26	TMAO	↑	↑	↓	↓	↑	↑
3.66	Choline	↓	↑	–	–	↓	↑
3.9	Hippurate	↓	↓	↓	↓	↓	↓

↑ indicates increase in signal; ↓, decrease in signal; –, loading not significant for that subject.

[a] Identified by 2D NMR as *N*-acetylglutamate, glutamine and glutamate signals.

9. Results and discussion

9.1. ^{1}H NMR spectral analysis

Visual analysis of the spectra showed that there were distinct variations between urine spectra obtained before and after soy isoflavone consumption, as well as intra-

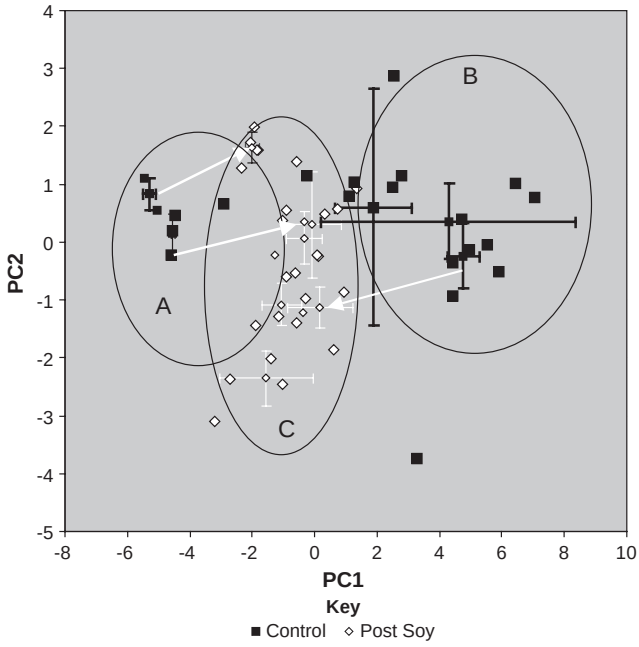

Fig. 2. PCA score plot based on MC descriptors derived from urinary ^{1}H NMR spectra. The plot illustrates the separation of spectral data based on intersubject variation (1–6) and variation related to diet — pre- (■) and post-soy (◇) sample. The PCs describe 79.4% of the variation in the data. Fig. 1 illustrates that there is initially two biochemically distinct pre-soy groups (A and B); on soy consumption the biochemical profile of these two groups focuses into one group (C). The ellipses represent individual with the arrow indicating their biochemical trajectory post-soy. The mean positions for samples pre- and post-soy along with the standard deviation are also plotted for each individual.

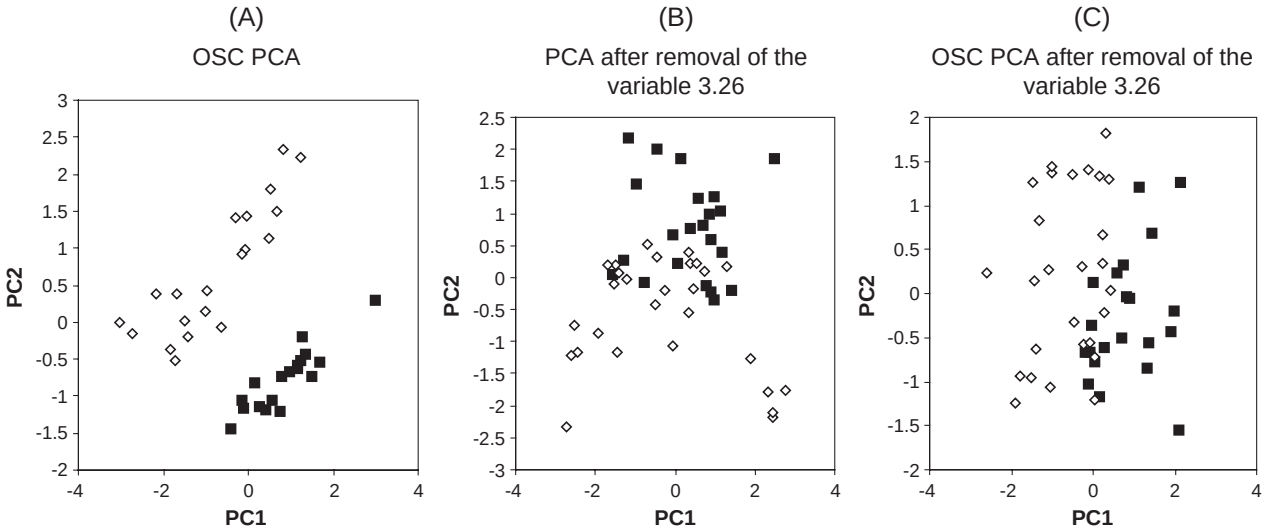

Fig. 3. The PC1 and PC 2 scores plot based on MC descriptors derived from urinary ^{1}H NMR spectra illustrates the separation between spectra collected from subjects pre- (■) and post-soy (◇). (A) PCA scores plot, based on *post OSC* MC descriptors. (B) PCA scores plot based on MC descriptors after elimination of the dominating variable (3.26). (C) PCA scores plot based on MC *post OSC* descriptors after elimination of the dominating variable (3.26).

sample variation due to the other intrinsic factors, such as menstrual cycle.

9.1.1. Miso intervention (unconjugated isoflavones)

PCA analysis of the spectral data generated from urine samples collected before and after miso intervention showed distinct separation in PC1 (Fig. 1A), suggesting that dietary intervention with miso (an unconjugated form of aglycone isoflavones) resulted in significant biochemical effects. Interrogation of the loadings (variables contribution plot) further indicated that the separation between intervention periods was mainly due to variable 3.26 (covering the spectral region 3.24–3.28 ppm). Further analysis of the spectra indicated that this variable was associated with a marked spectral increase in trimethlyamine-N-oxide (TMAO) signal at δ 3.27.

PCA carried out on the data after removal of the dominant variable 3.26 still revealed some evidence of clustering according to dietary intervention phases in PC1 vs. PC2 (Fig. 1B). However, the separation was not as pronounced as that observed before the removal of variable 3.26 (TMAO $-\delta$ 3.27; Fig. 1A), providing further evidence to suggest that the key biochemical marker relating to dietary intervention with miso was an increase in TMAO. For each of the three subjects, subtle variation in other metabolites was also identified (Table 1) and included an increase in methylamine, dimethylamine, choline, creatine, together with a modest decrease in citrate, lactate, creatinine and hippurate. Spectral investigation related these variables to changes in intermediates from the methylamine pathway and osmolytes, whose changes may be related directly to the effects of miso on urinary TMAO excretion. In addition, changes in metabolites associated with energy metabolism, specifically that of carbohydrate and lipid metabolism pathways, were observed.

9.1.2. Textured vegetable protein intervention (conjugated isoflavone glucosides)

The initial PCA also suggested that TVP had significant effects on the urinary profile. PCA of spectral data of samples collected during the TVP intervention study revealed three main clusters. The control group appeared to consist of two biochemically distinct groups A and B, following TVP consumption changes in the these two groups resulting in the formation of a third biochemically distinct profile group C, which suggests a spectral lensing[4] effect on the biochemical profile in urine following soy intervention (Fig. 2).

This response appears to be subject specific (Fig. 2), as each subject initially had a unique position in the multivariate space (A or B) pre-TVP intervention, but as a consequence of TVP consumption a uniformity of the spectral profile (mapping) of all subjects (within multivariate space C) was observed.

PCA conducted separately for each individual also illustrated that all subjects had a biochemical response to the soy diet (data not shown), and these effects were evident from changes in the urine profile.

Although there were several key variables common to all subjects as a response to soy (TVP) intervention (Table 1), as with miso consumption, the dominating variable exerting the greatest leverage on the models appeared to be 3.26 (metabolite signal for TMAO) that contributed to both variations between diets and subjects. However, analysis of the spectral variables for each individual demonstrated that the major effects of TVP were to some extent subject

[4] A 'metabolic lensing' effect is a result of a stressor or xenobiotic, a larger overall effect on biochemical status within a system, thereby causing two or more subpopulations to comap following intervention when compared with natural biochemical changes with time (Bailey N.J.).

specific (data not shown) in that the levels of response to the change in diet differed from subject to subject.

9.1.3. PC analysis of filtered NMR spectral data

To further validate these observed differences in the metabolic profile of urine following TVP intervention, OSC was applied to the TVP data set to eliminate intersubject variation and focus on the specific effects of dietary intervention.

PCA of these filtered data gave marked clustering of spectra based on soy intervention (Fig. 3A). The variable distribution plot revealed that the key loading contributing to this separation was 3.26. Spectral analysis suggested that this was related to an increase in TMAO following consumption of soy. In addition, on analysis of the removed orthogonal variables it appeared that variable 3.26 significantly contributed to intersubject variation. This was also evident in the initial PCA (Fig. 2) and analysis of loadings relating to dietary variation for each individual (Table 1). From these analyses it was clear that there were two basic classes of subject, those subjects whose levels of TMAO increased on soy intervention and those subjects whose TMAO levels decreased on soy intervention — group A and group B.

Variation in TMAO metabolism is present in the general population and is related to difference in the ability to metabolize trimethyamines (TMA) to TMAO in the liver. The inability to completely oxidize TMA to TMAO results in trimethylaminuria (TMAU) and is a metabolic disorder that produces pronounced body odors resulting from the systemic accumulation of an excessive amount of unoxidized trimethylamine that is then excreted in the breath, sweat and urine [17]. The biochemical deficit causing this condition is due to a range of genetic mutations in the flavin-containing monooxygenase 3 (FMO3) enzyme. However, the PCA indicated that the metabolic lensing effect observed is related to harmonization in the metabolism pathways affecting TMAO elimination, to attain a 'new' homeostatic balance (group C) that is independent of the normal homeostatic position of a specific individual. Furthermore, if subjects were displaying a metabolic disorder relating to TMA metabolism, levels of TMA in the urine would be higher in subjects excreting lower levels of TMAO, and thus TMA would be highlighted in the PC loadings in relation to intersubject variation.

9.1.4. PCA of data after removal of the dominating variable 3.26

In order to interrogate the significance of the dominating variable TMAO (δ 3.27) in relation to TVP intervention and intersubject variation, PCA of unfiltered data was conducted after elimination of this variable (3.26). PCA of these data resulted in marked separation between spectra collected before and after soy intervention in PC2 without the need for data filtration (Fig. 2B) and enabled the identification of the more subtle changes in the urinary biochemical composition contributing to dietary-based PCA separation. These variables were similar to those identified in Table 1 relating to dietary intervention with miso.

These analyses indicated that TMAO was the main variable contributing to intersubject variation, and that other variables were important contributors to the observed dietary-based separation.

To enhance the clustering of samples based on dietary intervention (TVP or control) following removal of the dominant variable 3.26, a spectral filter was applied to the data and PCA performed. Marked separation between spectra collected before and after intervention was observed in PC1 (Fig. 3C). The variables identified were not dissimilar to those identified before application of a filter, although separation was promoted to a higher PC indicating decreased extrinsic interference in the data.

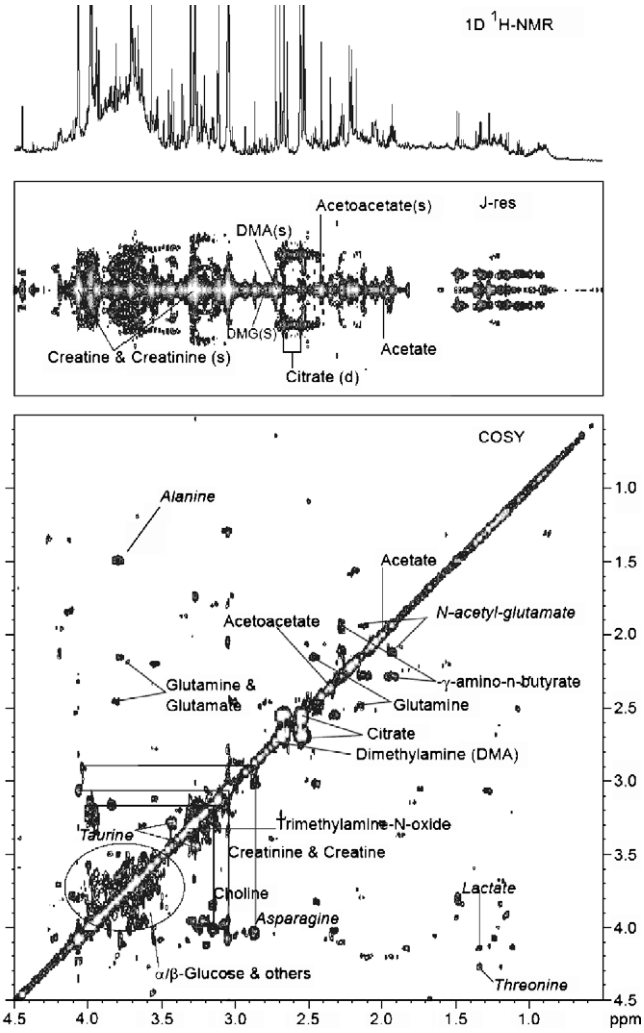

Fig. 4. Partial ^{1}H–^{1}H COSY spectra and J-res (δ 0–4.5.) of a urine sample collected after soy intervention. The metabolites highlighted are significant in the PCA separation of pre- and post-soy spectra, and thus are related to the biochemical effects of soy. Two-dimensional analysis of ^{1}H–^{1}H COSY spectra and J-res urine spectra enabled structural elucidation and thus identification and clarification of metabolite related to the effects of dietary soy.

Analysis of the spectral data, based on the PCA analysis and further 2D homonuclear ^{1}H–^{1}H COSY spectral analyses, along with spiking of authentic standards, allowed the identification of the key endogenous metabolites that changed following soy intervention (Fig. 4). Soy ingestion resulted in changes in endogenous methylamine, dimethylamine, trimethylamine-*N*-oxide, choline, glutamine and glutamate levels, with decreases in creatinine, along with a corresponding increase in creatine resonances and minor modification in TCA intermediates. There were also significant changes in the aromatic regions relating to hippurate and benzoate. The changes in the aromatic profile following soy consumption suggest potential changes in phenyl/benzoate metabolism, which may be due to isoflavone metabolites, modification in the gut-microflora following soy ingestion or endogenous changes in β-oxidation of fatty acids.

10. Discussion

These data suggest that dietary intervention with soy isoflavones, fed in either a conjugated or unconjugated form, results in increased urinary TMAO excretion. This modification in TMAO may lead to variations in the balance of several other osmolytes including betaine, choline, creatinine and creatine. These general effects of isoflavone-rich diets on osmolyte levels may indicate improved glomerular function or general kidney function. The modifications seen in kidney osmolyte activity due to dietary intervention with isoflavones may relate to the reported beneficial effects of soy on kidney function and hypertension. Short-term incorporation of soy protein in the diet (3 weeks) has been associated with lower renal plasma flow, glomerular filtration rate and fractional clearance of albumin [18]. The long-term effects of soy protein have yet to be fully understood. However, animal studies indicate that chronic soy protein intake preserves the function of damaged kidneys significantly better than animal protein [18]. It is suggested that incorporating soy into the diet will have therapeutic benefits in disease such as diabetic nephropathy by slowing deterioration of renal function and decreasing proteinuria [19–21].

However, the observed effects of soy on TMAO levels in urine may not be a specific effect of the isoflavone component of soy and may be related to the soy protein [21] or to the salt content, particularly of miso. High salt (Na^{+} Cl^{-}) can have significant effects on osmotic pressures and consequently affect levels of organic osmolytes. Increases in perturbing osmolytes such as salt (Na^{+} Cl^{-}) can result in the accumulation of nonperturbing osmolytes such as betaine.

An alternative explanation for the increased excretion of TMAO may lie with the gut microflora. Humans have coevolved with gut microflora and have a commensal relationship with many microorganisms in the gut, and the homeostatic balance and metabolic signature (metabolome) of an organism are dependent upon not only the host but the interaction between the host and its microfloral complement. Thus the urinary metabolite profile will be a product of this 'metabolome–metabolome' interaction [22]. Soy isoflavone metabolites such as equol, *O*-desmethylangolensin and the lignan enterolactone are not found in germ-free rats after the consumption of soy but may be found in germ-free rats associated with human flora [23]. Moreover, differences in the excretion of these metabolites in human populations can be reproduced in rats associated with human flora. For example, only approximately 35% of subjects excrete substantial amounts of equol after soy ingestion. Germ-free rats associated with flora from low-equol-producing subjects do not excrete equol, whilst those associated with high-equol-producing subjects excrete equol in considerable quantity [23]. It is likely that dietary intervention with soy TVP may influence the gut microbes, and as a consequence products of microbial metabolism such as choline, betaine and TMAO may be perturbed. Furthermore, intersubject variation in response to soy may reflect subject-specific differences in the gut microfloral species.

These analyses suggest that there were several processes, physiological and biochemical, that were modified on intervention with soya foods and result in a complex, mulitifactoral effect on TMAO excretion.

The complex biochemical picture relating to the effects of isoflavone-rich diets may relate in part to the wide range of mechanisms of action which have been ascribed to the isoflavones. In vitro investigations have demonstrated that isoflavones bind to oestrogen receptors (ER) with a preference for ERβ and also that they have a greater ability to trigger transcriptional function of ERβ [1,2], which suggests that these compounds may exert tissue-specific effects, but they also have numerous other biological effects independent of the ER (e.g., antioxidant capacity, antiproliferative and antiangiogenic effects) [1,2]. However, whether isoflavones have any biological activity in vivo, either hormonal or nonhormonal, is a contentious issue, and there is currently a paucity of data on their biological mechanisms of actions in vivo. Thus the biochemical effects observed using NMR spectroscopy on these controlled dietary intervention studies samples give a first insight into potential in vivo mechanisms of action.

Increases in the levels of the glucogenic amino acids glutamate and glutamine (illustrated in Fig. 4) (which are easily converted to tricarboxylic acid cycle intermediates) could be related to modifications in TCA cycle and/or increase in protein breakdown. The additional decreases in citrate concentration and changes in the sugar region imply a decrease in the rate of glycolysis and thus modification in carbohydrate metabolism (Table 1). Furthermore, the changes in endogenous methylamine pathway intermediates, and corresponding modification in the urine concentration of choline, betaine, glycine and acetate, suggest soy consumption generated changes in lipid and cholesterol metabolism and transport.

These results correlate with the reported changes in the plasma biochemical profile in response to dietary intervention with TVP [24]. In these data, key changes in the plasma profile, relating to lactate, lipoprotein and specific sugars, were determined. In addition, the response was to some extent subject specific and indicated that genetic variability may play a significant role in the health effects of a soy diet. Together, these results suggest an inhibitory effect of isoflavones on glycolysis, resulting in a general shift in energy metabolism from carbohydrate metabolism to that of lipid metabolism. An understanding of the underlying processes resulting in this overall biochemical picture and the biological implication of these changes may be gained to some extent from interrogation of reports relating to the general biological effects associated with oestrogenic compounds and oestrogen itself. Endogenous oestrogen is known to have a beneficial effect on lipid metabolism and plays a role in fat distribution in women [25]. In addition, it has been shown that isoflavones, i.e., rotenone, are mitochondrial complex I inhibitors [26] and therefore exert an inhibitory effect on oxidative metabolism and free radical production. This property may also be a feature of soy isoflavones. The multiple properties of isoflavones on various components may provide an explanation for the observed effects on energy metabolism pathways. Furthermore, they provide a biochemical relationship between the many health benefits of an isoflavone-rich diet in relation to CVD.

Comparative analysis of the effects of TVP and miso suggests that conjugated (TVP 45 mg) and unconjugated isoflavones (miso 25 mg) have a similar biological activity, but the magnitude of their effects differs, indicating that conjugation has a significant effect that may relate to bioavailability and metabolism.

Several recent studies have investigated the potential relationship between isoflavones in the glucoside and algycone form to assess the potential differential effects on the absorption, distribution, metabolism and excretion of isoflavones in animal and human studies [27–30]. However, some data reported that isoflavones aglycones were absorbed more efficiently than isoflavone glycosides, while other data suggest that the resulting bioavailability of daidzein and genitein was greater when the isoflavones were ingested as glucosides rather than as aglycones, and in a further study no difference in the bioavailability following consumption of aglycones or glucoside tablets was evident [27–30]. These reports do not provide a clear answer to the relationship between conjugation and bioavailability. From our analyses, it may be proposed that the difference in the efficacy of glucosides and aglycones is likely to be due to a number of factors, including differences in their metabolism and elimination which effect the efficacy of isoflavone metabolites.

From this study, it can be established that metabonomic technology is of value to the area of nutrition and health and for the analysis of human data. This study has demonstrated how biofluid NMR-based metabonomic studies can give information on the subtle biochemical effects of dietary components in a complex in vivo system like humans. It provides additional insight and knowledge of the mechanisms behind the biological activity of dietary components.

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