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A Metabonomic Strategy for the Detection of the Metabolic Effects of Chamomile (*Matricaria recutita* L.) Ingestion

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A metabonomic strategy, utilizing high-resolution ^{1}H NMR spectroscopy in conjunction with chemometric methods (discriminant analysis with orthogonal signal correction), has been applied to the study of human biological responses to chamomile tea ingestion. Daily urine samples were collected from volunteers during a 6-week period incorporating a 2-week baseline period, 2 weeks of daily chamomile tea ingestion, and a 2-week post-treatment phase. Although strong intersubject variation in metabolite profiles was observed, clear differentiation between the samples obtained before and after chamomile ingestion was achieved on the basis of increased urinary excretion of hippurate and glycine with depleted creatinine concentration. Samples obtained up to 2 weeks after daily chamomile intake formed an isolated cluster in the discriminant analysis map, from which it was inferred that the metabolic effects of chamomile ingestion were prolonged during the 2-week postdosing period. This study highlights the potential for metabonomic technology in the assessment of nutritional interventions, despite the high degree of variation from genetic and environmental sources.

KEYWORDS: NMR-based metabonomics; chamomile; *Matricaria recutita* L.; orthogonal signal correction

INTRODUCTION

Natural products, such as chamomile, have been employed as alternative medicines or functional foods, on a worldwide basis, for many centuries. The flower of chamomile is a common herbal beverage and is used as an anti-inflammatory, a mild sedative, and an antiulcer remedy. Chamomile has also been reported to have antioxidant activity (*1*), and the "essential oil" extracted from the chamomile flowers has been shown to possess antimicrobial activity (*2, 3*). To date, most of the studies on chamomile have focused on the characterization and assessment of the biological activity of individual extractable components. For example, chamazulene has been found to inhibit the formation of leukotriene B-4 in intact cells and may contribute to the anti-inflammatory activity of chamomile by inhibiting the leukotriene synthesis and additional antioxidative effects (*4*). Flavonoids isolated from chamomile, such as apigenin, have been shown to reduce γ-aminobutyric acid-activated chloride currents and also to reduce locomotor activity of rats (*5, 6*). However, little has been published on the response of human biological systems to the intake of chamomile tea. Here, we

apply a ^{1}H NMR-based metabonomic strategy to generate data on the global metabolic response to chamomile tea ingestion in man.

^{1}H NMR spectroscopy facilitates the detection of a wide range of low molecular weight metabolites commonly found in tissues and biofluids including urine and blood plasma. It typically generates thousands of resonance signals that can be related to the response of biological systems to perturbation of the system via pathological events, therapeutic intervention, or genetic modification and results in modulation of these signals, which can be related to the metabolic response of the organism. Due to the high density of the spectroscopic data, it is desirable to characterize these modulations by the application of multivariate statistical data analysis so as to reduce the complexity of these data and to facilitate visualization of inherent patterns in the data. Various multivariate statistical data analyses, including projection methods such as principal components analysis (PCA) and projection to latent structures (PLS) based methods (*7, 8*), Bayesian probabilistic approaches, and neural networks (*9*), have been extensively applied to biomedical problems including the characterization of human urine samples from patients with inborn errors of metabolism (*10*), investigation of the biochemical consequences of genetic strain differences in mice (*11*) and to the study of the biological system response to drugs (*12, 13*) or other xenobiotics (*11*).

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In contrast to the generally striking perturbations induced by drugs or diseases, NMR profiles of biofluids also contain latent information on the more subtle consequences of diet and physiological variation, such as diurnal cycles, hormonal status, and gender differences (*14*). In addition, the gut microbial communities contribute to the overall metabolite profile, and changes to the gut microbiotia can be readily observable in the spectroscopic profiles (*15*). In cases when metabolic response to toxicity, diseases, or drugs is the primary focus of interest, it is not often necessary to take the subtle physiological variation into consideration because the metabolic response to the pathology intervention will eclipse minor metabolic variations due to physiological variations. However, in human studies, more subtle variations introduced by variations in diet or local environment become important confounding factors when metabolic response to nutritional intervention is studied. To extract meaningful biological information from data confounded by diverse extraneous physiological variation, data-filtering methods have been employed. One of the most frequently used data-filtering methods is orthogonal signal correction (OSC) (*16*). This method has been successfully applied to aid the diagnosis of coronary heart disease (*17*), to remove variation due to instrument and physiological variations (*18*), and to facilitate the detection of strain-related differences in mouse urine by removal of the variation due to diurnal cycle (*19*). The advantage of applying OSC to ^{1}H NMR spectroscopic data is that the variation not correlated to the biological response of interest is removed and the subsequent PCA or PLS models calculated are focused solely on discriminating between elected classes (*18*).

In this study, we investigated the response of human volunteers to chamomile intake over a 6-week period incorporating a baseline, a treatment, and a post-treatment period. A metabonomic strategy, which combines ^{1}H NMR spectroscopy and multivariate data analysis together with OSC, has been applied to extract information regarding the human metabolic response to the ingestion of chamomile tea.

MATERIALS AND METHODS

Materials. A batch of chamomile flowers obtained from Slovakia was authenticated by Eurofins Scientific Ltd (C200203M2431) and deposited in the herbarium at Kings College London, coded Ma12-1-J9. The quality of the material was assessed to ensure that it met the requirements of the European Union pharmacopoeia. Approximately 4 kg of chamomile flowers was mixed to produce a homogeneous sample, and a representative portion (1 kg) was then ground into a fine powder and sieved through a 2 mm sieve. The chamomile tea was prepared by infusing 5 g of powder in 200 mL of boiling water for 10 min followed by straining, corresponding to a daily intake of ~5 cups of tea from commercial preparations.

Urine Sample Collection Details. Fourteen healthy male ($n = 7$) and female volunteers ($n = 7$) were enrolled in the study, and written consent was obtained from each volunteer. The study consisted of three phases. The first 2 weeks of the study was the control period, and spot urine samples were taken from the volunteers between 11:30 and 11:50 a.m. This was followed by a 2-week "dosing" period and a 2-week "postdosing" period. During the dosing period, volunteers were given 200 mL of chamomile tea each day at approximately 10:00 a.m., and urine samples were collected from these volunteers later the same day between 11:30 and 11:50 a.m. A total of 420 urine samples were collected for this study: 140 samples from the control or preingestion period; 140 samples for the dosing period; 140 samples for the post-treatment period. The samples of the post-treatment period were collected in the same manner as during the first 2 weeks. Volunteers reported no adverse effects after ingestion of chamomile tea. Food restrictions were not applied, but dietary information from the preceding

evening and morning prior to sample collection was recorded to establish potential confounding dietary effects manifested in the spectra. These effects were taken into account during subsequent data analysis. The urine samples were stored frozen at -40 °C prior to the NMR spectroscopic experiments.

^{1}H NMR Spectroscopy of Urine Samples. Urine samples were prepared by mixing 400 μL of urine with 200 μL of phosphate buffer containing 0.2 M NaH_2PO_4 and 0.8 M Na_2HPO_4, 10% D_2O as a field lock, and 0.05% sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 as a chemical shift reference (pH 7.4). ^{1}H NMR spectra were recorded on a Bruker DRX 600 NMR spectrometer, operating at 600.13 MHz for ^{1}H, equipped with a 5 mm flow probe. A standard one-dimensional pulse sequence (RD$-90°-t_1-90°-t_m-90°-$ACQ) was employed (*20*). Water suppression was achieved with weak irradiation on the water peak during the recycle delay (RD = 2 s) and mixing time, t_m, of 150 ms; t_1 was set to 3 μs. The 90° pulse length was adjusted to ~10 μs. Sixty-four transients were collected into 32K data points for each spectrum with a width of 20 ppm and a total repetition time of 3.8 s.

Data Reduction and Pattern Recognition. NMR spectra were corrected for phase and baseline distortions using a MATLAB script, NMR*proc*, developed in-house (Dr. Tim Ebbels, Imperial College). All free induction decays were multiplied by an exponential window function with a 1 Hz line-broadening factor prior to Fourier transformation. The spectra over the range δ 0.2$-$10.0 were divided into 245 regions, each 0.04 ppm wide, using AMIX (Bruker Analytik GmbH, Rheinstetten, Germany), and the signal intensity in each region was integrated. The region δ 4.5$-$6.4 was removed to eliminate the effects of imperfect water suppression. Citrate peaks at δ 2.72 and 2.68 were combined into a single peak (δ 2.7), and likewise the citrate resonances at δ 2.56 and 2.52 were combined into a single peak (δ 2.54) to take into account pH-related peak shifts. Furthermore, peaks at δ 4.04 and 4.08 were combined to avoid the creatinine resonance at δ 4.06 being divided across two consecutive integral regions. The integration of each region was normalized to the total sum of the spectral integral prior to pattern recognition analysis (PR) in order to partially compensate for differences in urine concentration. Multivariate data analyses including PCA, PLS, and OSC were carried out with the software Simca-P 8.0 (Umetrics, Umeå, Sweden). Each principal component (PC) is a linear combination of the original variables, whereby each successive PC explains the maximum amount of variance possible in the dataset and each PC is orthogonal to every other PC (*21*). Data were visualized by plotting the PC scores where each coordinate in the scores plot represents an individual sample and the PC loadings where each point represents one spectral region. Thus, the loadings plot gives an indication of the spectral variables of metabolites that most strongly influence the patterns in the scores plot. Similarities and differences between samples can be detected in the scores plots, whereas spectral regions responsible for the differences can be viewed in the corresponding loading plots. The PLS model was cross-validated using half of the dataset (urine obtained on alternate days) as a training dataset to calculate the OSC-filtered PLS model. The remaining half of the data were used as a test dataset to verify the predictability and robustness of the model. To decide the class membership of the training and predicted dataset, a class prediction value of 0.5 was used as a cutoff value for the two classes. For example, supposing the samples from before and after chamomile ingestion are regarded as "1" and "0", respectively; if the prediction value is above 0.5 for a sample before chamomile ingestion, then the sample is regarded as being correctly predicted.

RESULTS AND DISCUSSION

^{1}H NMR Spectra of Human Urine Samples. ^{1}H NMR spectra of urine obtained from a male volunteer before and during chamomile intake are shown in **Figure 1**. The resonance assignments were made according to the literature (*20*) and on the basis of two-dimensional ^{1}H$-^1$H correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY) NMR spectra. Visual inspection of paired spectra revealed a similarity in overall biochemical composition between urines obtained
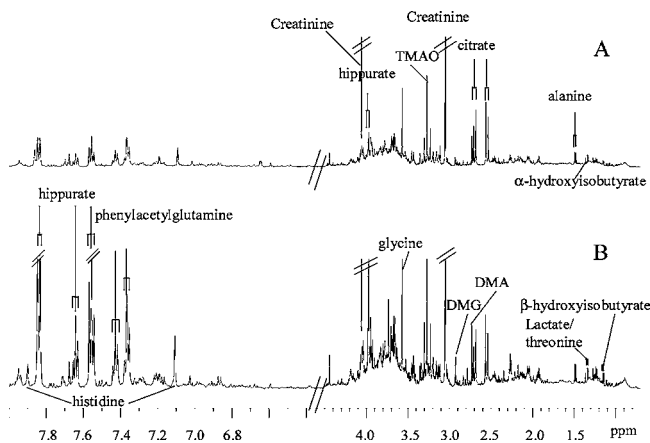
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Figure 1. 600 MHz ¹H NMR spectra of urine sample obtained from a healthy male (**A**) before chamomile intake and (**B**) during chamomile intake. Aromatic regions of spectra were magnified 4 times compared to the corresponding aliphatic regions.
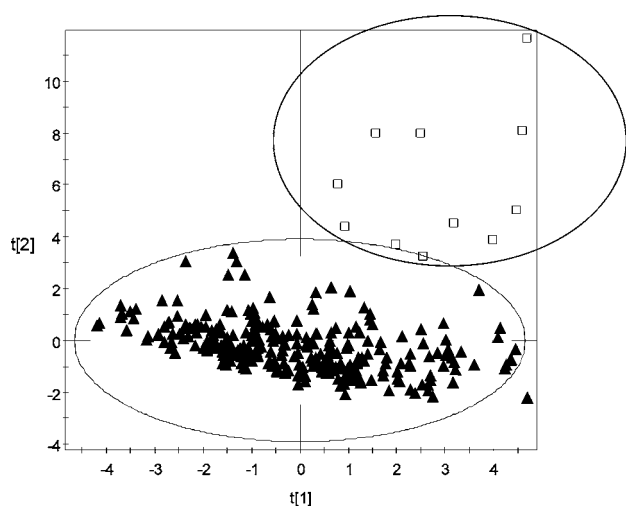

Figure 3. PCA scores plots (t[1] vs t[2]) of ¹H NMR spectra from control human urine showing the variations due to gender: (■) male; (□) female.

Figure 2. Scores plots (t[1] vs t[2]) generated from PCA of ¹H NMR spectra from control human urine showing the effect of fish ingestion: (□) outliers from those who had fish within ~16 h prior to sampling.

before and during chamomile ingestion, although a number of metabolite resonances were present in higher intensity in the urine obtained after chamomile intake. These resonances were assigned to alanine, dimethylamine, trimethylamine-*N*-oxide, creatinine, and hippurate. However, due to the strong influence of inter- and intraindividual variation and particularly with regard to the fact that the experiment was carried out without dietary control, the biological responses to chamomile tea intake are more effectively monitored by multivariate data analysis of alteration in spectral profiles.

Dietary Effects on Human Urinary Metabolites. PCA was initially carried out on the whole dataset using mean-centered data to establish the presence of any chamomile-related metabolic changes. The PCA scores plot calculated from the whole dataset revealed several outlying samples (□) deviating from the rest of the data (▲) in the second principal (t[2]) direction based on the Hotelling 95% confidence ellipse (**Figure 2**). Inspection of the corresponding loadings plots showed that increased resonance intensity for the δ 3.26−3.30 regions, corresponding to an elevation in the trimethylamine-*N*-oxide resonance, was predominately responsible for this deviation. It has been reported that a diet with a high content of fish leads to an increase in trimethylamine-*N*-oxide excretion in urine (*22*). Trimethylamine-*N*-oxide is an organic osmolyte present at high
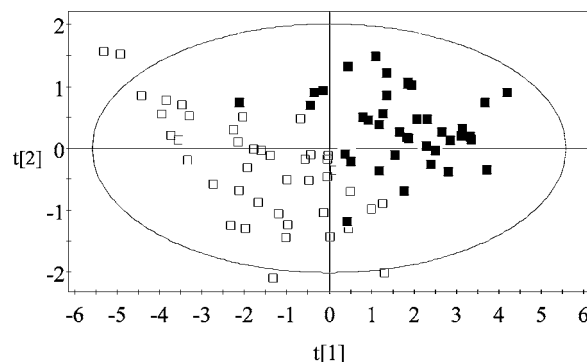
levels in fish, particularly deep-dwelling fish (*23*). An inspection of the diet diaries confirmed the link between fish consumption and increased urinary excretion of trimethylamine-*N*-oxide. These samples exerted a high leverage on the dataset and were removed subsequently from further analysis.

Gender Differences in Urinary Metabolites. After the outlying samples were removed, the PC scores plot indicated the presence of clusters (**Figure 3**). The sample population largely divided according to gender in the first principal component (t[1]). By inspection of the corresponding PC loading and with the ¹H NMR spectra of urine samples from female and male volunteers, it was clear that this gender-based separation was attributed to relatively higher levels of citrate and glycine in female urines compared to male urines. The higher concentration of citrate in female humans has been observed previously and was reported to be originated from blood and epithelial cells (*24*) and was suggested to relate to female estrogen levels and the female reproduction system (*25*). High levels of glycine in female urine have not been reported previously, However, glycine was found to be of great importance in early gestation (*26*) and was the most abundant amino acid in oviductal, uterine (*27*), and allantoic fluids (*28*) in female animals. Therefore, the higher levels of glycine in female urine may be related to activity of the female reproduction system. Gender differences in urinary profiles were also found in the Han Wistar (HW) rat. These were mainly manifested in high urinary excretion of the dimethyamine and trimethylamine-*N*-oxide in female rats, partly due to the effect of estrogen and testosterone on choline metabolism (*29*). In addition, the level of creatinine was found to be higher in urine from male participants compared with female. Creatinine is an index of muscle mass and was found to be directly correlated with body weight (*30*). Because male volunteers are generally heavier than females in body mass, it is unsurprising that creatinine concentration was higher in male urine.

Effects of Chamomile Intake. Human urinary metabolites were shown to be affected by dietary variation and gender as discussed above. In addition, ¹H NMR-based metabonomic studies on human urine obtained under different physiological conditions such as pre- and postexercise have also shown interperson variability (*10*, *18*). All of these variations in metabolic profile can obscure the effect of specific dietary interventions such as the focus of interest in this study, that is, the metabolic effects of chamomile intake. To establish metabolic changes relating to chamomile, it is necessary to take the confounding factors into account prior to data analysis. OSC data filtration was applied to the dataset with a view to removing some of the extraneous influence of variation in urinary composition. Following the application of OSC, separation of
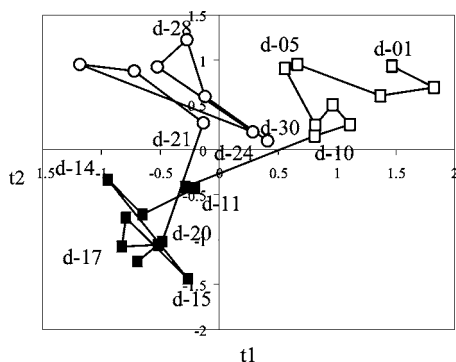
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Figure 4. Trajectory of PLS scores plot of t[1] versus t[2] for ¹H NMR data after orthogonal signal correction showing the dynamic progression of the urinary metabolite composition and highlighting the separation between, before (□), during (■), and after (○) chamomile tea ingestion. d indicates the day of sampling, where days d01–d10 represent the pre-chamomile phase, d11–d20 the period of chamomile ingestion, and d21–d30 the recovery or post-chamomile phase.
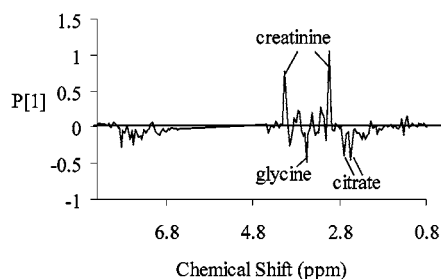

Figure 5. Loading plot of the excluded orthogonal component related to interperson variations.

the urine samples obtained from pre-, during, and post-chamomile tea ingestion was revealed in the trajectory of PLS scores plot (**Figure 4**). A PLS model was constructed with five significant PLS components using Pareto scaled data ($1/\sqrt{SD}$) and three *Y* variables corresponding to the sampling periods. After data filtration, in which one orthogonal component containing 18% of total variation was removed, clustering of samples on the basis of chamomile ingestion was achieved. The excluded orthogonal component predominantly related to interperson variation in metabolite profiles (**Figure 5**), which hitherto obscured the chamomile-induced changes in the spectral profile. Metabolites such as citrate, glycine, and creatinine were subject to strong interperson variation in urinary profiles.

The model characterizing the effects of chamomile ingestion was cross-validated to ascertain the validity of the separation shown in **Figure 4**. In addition, using an independent test set, in which the probability of a sample belonging to the class "1" is >0.01, 86% of samples were correctly predicted for the samples obtained during the period of chamomile ingestion. Having validated the model, it was then possible to analyze the biological response to chamomile intake.

The dominant metabolites influencing the differentiation between control urine samples and urine samples collected during the period when volunteers ingested chamomile tea are listed in **Table 1** together with a measure of their relative influence on the model given by the variable importance parameter. The largest influence on the separation between the controls and chamomile ingestion was increased urinary excretion of hippurate, glycine, and an unknown metabolite at δ 6.4, 6.44, 6.8 (UN), as well as decreased urinary excretion of creatinine (**Figure 4** and **Table 1**). Urinary hippurate results from both renal and hepatic synthesis of glycine and benzoic

Table 1. Changes of Metabolites Observed in Human Urines Obtained after Chamomile Ingestion and the Importance of Contributions from Each Metabolite

metabolite	chemical shift (δ)	change direction[a]	relative importance of contribution (rank)
creatinine	4.06	↓	5.226 (1)
	3.04		2.420
glycine	3.52	↑	2.637 (2)
hippurate	7.56		2.504 (3)
	3.96	↑	2.405
	7.64		2.401
	7.84		2.360
unknown	6.8		2.485 (4)
	6.44	↑	2.419
	6.4		2.396

[a] Key: ↓ indicates decrease and ↑ increase in the concentration of urines after chamomile ingestion.

acid. It is also the degradation product of flavonols due to the activity of intestinal microoganisms (*31*). In a study of human black tea consumption, the amount of hippurate excretion was found to be positively correlated with polyphenol intake (*32*). Because chamomile contains high levels of phenolic compounds, the increase of urinary hippurate excretion after chamomile ingestion was potentially due to the effect of polyphenols. The peaks at δ 6.4, 6.44, and 6.8 were elevated after chamomile ingestion, and inspection of the ¹H NMR spectrum of the chamomile tea suggested that the elevation of the unknown metabolite might originate directly from the chamomile tea. The nature of the metabolite is under investigation.

Although creatinine and glycine were subjected to high degrees of intersubject variation, much of this effect was removed by the filtration, and therefore the decrease in urinary excretion of creatinine and the increase in glycine observed after chamomile ingestion revealed in the PLS model was found to be a consistent human metabolic response to chamomile intake. This observation has not been reported in the literature. The mechanism of the changes of creatinine and glycine observed is unclear. However, it is known that oxidative stress promotes urinary excretion of creatinine (*33*, *34*), and studies on the effects of antioxidant on rabbits showed decreased levels of urinary creatinine (*35*). It is therefore possible that the reduced level of urinary creatinine following chamomile tea intake resulted from the antioxidative activity of chamomile.

The urine samples obtained from the 2 weeks of post-treatment period were isolated from these obtained from both the treatment and the pretreatment phases (**Figure 4**), suggesting an incomplete recovery after a 2-week period of chamomile ingestion. The corresponding loading plot indicated elevated urinary hippurate in those samples obtained during the post-treatment period. The concentration of hippurate and other metabolic species in urine has been shown to be modulated according to microbes. For example, Nicholls et al. (*15*) reported that the level of hippurate excretion in rats largely depended on the gut microbiotial colony and that establishment of stable gut microbiotia was achieved at 21 days after germ-free rats had been introduced into a standard laboratory environment. In other studies hippurate and 3-hydroxyphenylpropionic acid excretions were shown to be correlated with the microfloral composition of the colon (*36*). Because chamomile flowers have been shown to possess antimicrobial activity (*2*, *3*), a possible explanation for the observed incomplete recovery after 2 weeks of treatment has ceased would be that disruption of the gut microbiotial colony had occurred due to the antimicrobial

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activity of the chamomile-induced perturbation in the metabolic profiles and that >2 weeks was required for the reestablishment of gut microbiotia after chamomile ingestion. It is known that gut microbiotia exert a profound impact on the development and structure of the intestinal epithelium, the digestive and absorptive capabilities of the intestine, and the host immune system (*37*). Obviously disturbances of gut microbiotial by chamomile ingestion would be expected to have an impact on health. Microbiological identification of changes of specific changes in the microbiotial community would be helpful in addressing the metabolic implications associated with chamomile ingestion.

These results and observations highlight the diversity of physiological variation in human metabolism and emphasize the problems in studying metabolic response to nutritional intervention in small population studies. NMR-based metabonomics together with OSC and PLS modeling provided a means of extracting information related to dietary intervention with chamomile. The PLS model reflecting the human metabolic response to chamomile intake was validated by successful prediction of class membership of a test dataset with an accuracy of 86%. It was found that the depletion of creatinine and the elevation of hippurate, glycine, and other molecules were strongly associated with chamomile intake. In addition, the metabolic consequences of chamomile ingestion were prolonged in the 2-week post-dosing period, implying a persistent disruption of the resident gut microflora activities.

ABBREVIATIONS USED

PCA, principal components analysis; OSC, orthogonal signal correction; PLS, projection to latent structures.

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LITERATURE CITED

(1) Bandoniene, D.; Pukalskas, A.; Venskutonis, P. R.; Gruzdiene, D. Preliminary screening of antioxidant activity of some plant extracts in rapeseed oil. *Food Res. Int.* **2000**, *33*, 785−791.

(2) Marino, M.; Bersani, C.; Comi, G. Impedance measurements to study the antimicrobial activity of essential oils from *Lamiaceae* and *Compositae*. *Int. J. Food Microbiol.* **2001**, *67*, 187−195.

(3) Modesto, A.; Lima, K. C.; de Uzeda, M. Effects of solutions used in infants' oral hygiene on biofilms and oral microorganisms. *J. Dent. Child.* **2000**, *67*, 338−342.

(4) Safayhi, H.; Sabieraj, J.; Sailer, E. R.; Ammon, H. P. T. Chamazulene—an antioxidant-type inhibitor of leukotriene B-4 formation. *Planta Med.* **1994**, *60*, 410−413.

(5) Avallone, R.; Zanoli, P.; Puia, G.; Kleinschnitz, M.; Schreier, P.; Baraldi, M. Pharmacological profile of apigenin, a flavonoid isolated from *Matricaria chamomilla*. *Biochem. Pharmacol.* **2000**, *59*, 1387−1394.

(6) Zanoli, P.; Avallone, R.; Baraldi, M. Behavioral characterisation of the flavonoids apigenin and chrysin. *Fitoterapia* **2000**, *71*, S117−S123.

(7) Holmes, E.; Nicholls, A. W.; Lindon, J. C.; Ramos, S.; Spraul, M.; Neidig, P.; Connor, S. C.; Connelly, J.; Damment, S. J. P.; Haselden, J.; Nicholson, J. K. Development of a model for classification of toxin-induced lesions using ¹H NMR spectroscopy of urine combined with pattern recognition. *NMR Biomed* **1998**, *11*, 235−244.

(8) Tate, A. R.; Foxall, P. J. D.; Holmes, E.; Moka, D.; Spraul, M.; Nicholson, J. K.; Lindon, J. C. Distinction between normal and renal cell carcinoma kidney cortical biopsy samples using pattern recognition of ¹H magic angle spinning (MAS) NMR spectra. *NMR Biomed.* **2000**, *13*, 64−71.

(9) Holmes, E.; Nicholson, J. K.; Tranter, G. Metabonomic characterization of genetic variations in toxicological and metabolic responses using probabilistic neural networks. *Chem. Res. Toxicol.* **2001**, *14*, 182−191.

(10) Holmes, E.; Foxall, 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 nuclear-magnetic-resonance spectra of human urine from normal and pathological states. *Anal. Biochem.* **1994**, *220*, 284−296.

(11) Gavaghan, C. L.; Holmes, E.; Lenz, E.; Wilson, I. D.; Nicholson, J. K. An NMR-based metabonomic approach to investigate the biochemical consequences of genetic strain differences: application to the C57BL10J and Alpk: ApfCD mouse. *FEBS Lett.* **2000**, *484*, 169−174.

(12) Holmes, E.; Nicholls, A. W.; Lindon, J. C.; Connor, S. C.; Connelly, J. C.; Haselden, J. N.; Damment, S. J. P.; Spraul, M.; Neidig, P.; Nicholson, J. K. Chemometric models for toxicity classification based on NMR spectra of biofluids. *Chem. Res. Toxicol.* **2000**, *13*, 471−478.

(13) Wang, Y.; Bollard, M. E.; Keun, H.; Antti, H.; Beckonert, O.; Ebbels, T. M.; Lindon, J. C.; Holmes, E.; Tang, H.; Nicholson, J. K. Spectral editing and pattern recognition methods applied to high-resolution magic-angle spinning ¹H nuclear magnetic resonance spectroscopy of liver tissues. *Anal. Biochem.* **2003**, *323*, 26−32.

(14) Bollard, M. E.; Holmes, E.; Lindon, J. C.; Mitchell, S. C.; Branstetter, D.; Zhang, W.; Nicholson, J. K. Investigations into biochemical changes due to diurnal variation and estrus cycle in female rats using high-resolution ¹H NMR spectroscopy of urine and pattern recognition. *Anal. Biochem.* **2001**, *295*, 194−202.

(15) Nicholls, A. W.; Mortishire-Smith, R. J.; Nicholson, J. K. NMR spectroscopic-based metabonomic studies of urinary metabolite variation in acclimatizing germ-free rats. *Chem. Res. Toxicol.* **2003**, *16*, 1395−1404.

(16) Wold, S.; Antti, H.; Lindgren, F.; Ohman, J. Orthogonal signal correction of near-infrared spectra. *Chemom. Intell. Lab.* **1998**, *44*, 175−185.

(17) Brindle, J. T.; Antti, H.; Holmes, E.; Tranter, G.; Nicholson, J. K.; Bethell, H. W. L.; Clarke, S.; Schofield, P. M.; McKilligin, E.; Mosedale, D. E.; Grainger, D. J. Rapid and noninvasive diagnosis of the presence and severity of coronary heart disease using ¹H NMR-based metabonomics. *Nat. Med.* **2002**, *8*, 1439−1444.

(18) Beckwith-Hall, B. M.; Brindle, J. T.; Barton, R. H.; Coen, M.; Holmes, E.; Nicholson, J. K.; Antti, H. Application of orthogonal signal correction to minimise the effects of physical and biological variation in high resolution ¹H NMR spectra of biofluids. *Analyst* **2002**, *127*, 1283−1288.

(19) Gavaghan, C. L.; Wilson, I. D.; Nicholson, J. K. Physiological variation in metabolic phenotyping and functional genomic studies: use of orthogonal signal correction and PLS-DA. *FEBS Lett.* **2002**, *530*, 191−196.

(20) Nicholson, J. K.; Foxall, P. J. D.; Spraul, M.; Farrant, R. D.; Lindon, J. C. 750-MHz ¹H and ¹H−¹³C NMR spectroscopy of human blood-plasma. *Anal. Chem.* **1995**, *67*, 793−811.

(21) Wold, S.; Esbensen, K.; Geladi, P. Principal component analysis. *Chemom. Intell. Lab.* **1987**, *2*, 37−52.

(22) Alwaiz, M.; Mitchell, S. C.; Idle, J. R.; Smith, R. L. The relative importance of N-oxidation and N-demethylation in the metabolism of trimethylamine in man. *Toxicology* **1987**, *43*, 117−121.

(23) Yancey, P. H.; Fyfe-Johnson, A. L.; Kelly, R. H.; Walker, V. P.; Aunon, M. T. Trimethylamine oxide counteracts effects of hydrostatic pressure on proteins of deep-sea teleosts. *J. Exp. Zool.* **2001**, *289*, 172−176.

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(24) Zuppi, C.; Messana, I.; Forni, F.; Rossi, C.; Pennacchietti, L.; Ferrari, F.; Giardina, B. ¹H NMR spectra of normal urines: Reference ranges of the major metabolites. *Clin. Chim. Acta* **1997**, *265*, 85−97.

(25) Hodgkinson, A. Citric acid excretion in normal adults and in patients with renal calculus. *Clin. Sci.* **1962**, *23*, 203−205.

(26) Vallee, M.; Guay, F.; Beaudry, D.; Matte, J.; Blouin, R.; Laforest, J. P.; Lessard, M.; Palin, M. F. Effects of breed, parity, and folic acid supplement on the expression of folate metabolism genes in endometrial and embryonic tissues from sows in early pregnancy. *Biol. Reprod.* **2002**, *67*, 1259−1267.

(27) Iritani, A.; Sato, E.; Nishikawa, Y. Secretion rates and chemical composition of oviduct and uterine fluid in sows. *J. Anim. Sci* **1974**, *39*, 582−588.

(28) Vanwinkle, L. J.; Haghighat, N.; Campione, A. L. Glycine protects preimplantation mouse conceptuses from a detrimental effect on development of the inorganic-ions in oviductal fluid. *J. Exp. Zool.* **1990**, *253*, 215−219.

(29) Stanley, E. G. ¹H NMR spectrascopic and chemometric studies on endogenous physiological variation in rats. Ph.D. Thesis, University London, 2002.

(30) Davies, K. M.; Heaney, R. P.; Rafferty, K. Decline in muscle mass with age in women: A longitudinal study using an indirect measure. *Metabolism* **2002**, *51*, 935−939.

(31) Rechner, A. R.; Kuhnle, G.; Hu, H. L.; Roedig-Penman, A.; van den Braak, M. H.; Moore, K. P.; Rice-Evans, C. A. The metabolism of dietary polyphenols and the relevance to circulating levels of conjugated metabolites. *Free Radical Res.* **2002**, *36*, 1229−1241.

(32) Clifford, M. N.; Copeland, E. L.; Bloxsidge, J. P.; Mitchell, L. A. Hippuric acid as a major excretion product associated with black tea consumption. *Xenobiotica* **2000**, *30*, 317−326.

(33) Almar, M.; Villa, J. G.; Cuevas, M. J.; Rodriguez-Marroyo, J. A.; Avila, C.; Gonzalez-Gallego, J. Urinary levels of 8-hydroxydeoxyguanosine as a marker of oxidative damage in road cycling. *Free Radical Res.* **2002**, *36*, 247−253.

(34) Okamura, K.; Doi, T.; Hamada, K.; Sakurai, M.; Yoshioka, Y.; Mitsuzono, R.; Migita, T.; Sumida, S.; SugawaKatayama, Y. Effect of repeated exercise on urinary 8-hydroxy-deoxyguanosine excretion in humans. *Free Radical Res.* **1997**, *26*, 507−514.

(35) Lee, J. Y.; Lott, J. A.; Kauffman, E. M.; Sharma, H. M. Effect of the herbal mixture MAK-4 on organ functions in WHHL rabbits. *Biochem. Arch.* **1997**, *13*, 285−296.

(36) Williams, R. E.; Eyton-Jones, H. W.; Farnworth, M. J.; Gallagher, R.; Provan, W. M. Effect of intestinal microflora on the urinary metabolic profile of rats: a H-1-nuclear magnetic resonance spectroscopy study. *Xenobiotica* **2002**, *32*, 783−794.

(37) Xu, J.; Gordon, J. I. Honor thy symbionts. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 10452−10459.