Study of Metabolic Profiling Changes in Colorectal Cancer Tissues Using 1D 1H HR-MAS NMR Spectroscopy

Siwon Kim, Sangmi Lee, Young Hee Maeng,[†] Weon Young Chang,[†] Jin Won Hyun,[†,*] and Suhkmann Kim[*]

Department of Chemistry and Chemistry Institute for Functional Materials, Pusan National University, Busan 609-735, Korea
[*]E-mail: suhkmann@pusan.ac.kr
[†]School of Medicine, Jeju National University, Jeju 690-756, Korea. [*]E-mail: jinwonh@jejunu.ac.kr

Metabolomics is a field that studies systematic dynamics and secretion of metabolites from cells to understand biological pathways based on metabolite changes. The metabolic profiling of intact human colorectal tissues was performed using high-resolution magic angle spinning (HR-MAS) NMR spectroscopy, which was unnecessary to extract metabolites from tissues. We used two different groups of samples, which were defined as normal and cancer, from 9 patients with colorectal cancer and investigated the samples in NMR experiments with a water suppression pulse sequence. We applied target profiling and multivariative statistical analysis to the analyzed 1D NMR spectra to identify the metabolites and discriminate between normal and cancer tissues. Cancer tissue showed higher levels of arginine, betaine, glutamate, lysine, taurine and lower levels of glutamine, hypoxanthine, isoleucine, lactate, methionine, pyruvate, tyrosine relative to normal tissue. In the OPLS-DA (orthogonal partial least square discriminant analysis), the score plot showed good separation between the normal and cancer groups. These results suggest that metabolic profiling of colorectal cancer could provide new biomarkers.

Key Words : HR-MAS NMR, Colorectal cancer, Metabolomics, Multivariate statistical analysis

Introduction

Colorectal cancer has recently been reported to be the second most common malignancy and is a leading cause of cancer-associated death in many countries.[1,2] Colorectal cancer is also one of the most leading causes of cancer morbidity in South Korea, accounting for more than 20,000 new cases in 2007. Colorectal cancer has been reported as the second most common cancer in males and the third most common cancer in females. The age-standardized rate for morbidity from colorectal cancer over the period from 2003 to 2007 was 18.1 per 100,000 per year in males and 11.3 in females. Colorectal cancer was the most-increased cause of cancer death over a 25-year period, with a 4.8-fold increase observed in males and a 3.6-fold increase observed in females. Similarly, the incidence of cancer also increased by 43% in males and 32% in females. According to the 2009 OECD Health at a Glance report, the rate of increase in the mortality of colorectal cancer in the nation was among the highest in the OECD. For colorectal cancer, the rate was similar to the average rate, *i.e.*, 59.1% in males (OECD average 56.4%) and 57.1% in females (OECD average 58%). Most of symptoms associated with colorectal cancer do not manifest until late in the process. Thus, a simple, non-invasive way of detecting the cancer at an early stage is important for the success of therapeutic interventions.

Metabolomics is a useful method to identify small molecules and metabolic pathways.[3-5] Metabolomics is the study of all naturally occurring small molecules, called metabolites, in biological samples such as cells, biofluids, or tissues.

These small molecules are the products of metabolic processes and are include various compounds such as sugars (*i.e.*, carbohydrates), fats (*i.e.*, lipids), nucleic acids, and amino acids.[6] The collection of all metabolites within a cell is called the metabolome. Various analytical technologies have been used for metabonomics research, but NMR spectroscopy and mass spectroscopy are mainly utilized to identify the hundreds of metabolites in the complex mixtures of the sample. In particular, NMR-based metabolomics has been applied in several studies for the diagnosis of diseases using different types of sample including body fluids, tissue extracts, and intact tissue. The use of NMR spectroscopy for metabolomics studies has several advantages including relatively high degree of reproducibility, easy-to-identify metabolites, facile determination of the structure of unknown metabolites, high throughput, and nondestructive sample treatment.[7] However, NMR has lower sensitivity than other analytic tools such as mass spectrometry.

In the past decade, studies have increasingly utilized high-resolution NMR spectroscopy for the research of biosystems. Body fluids such as blood,[8] urine,[9-11] serum,[12-14] cerebrospinal fluid (CSF).[15,16] are widely used for verifying metabolic mechanisms (or pathways) for many diseases. Recently, the NMR method was expanded to intact tissue extracted from animals and humans. The method to examine the intact tissue samples was developed from a solid-state NMR technique and is called high-resolution magic angle spinning (HR-MAS) NMR spectroscopy. HR-MAS NMR spectroscopy is a useful tool for analyzing intact tissues[2,17-19] that has been used for several years to discriminate among differ-

ent states (conditions) of tissue and cell lines. The line broadening effects of chemical shift anisotropy and dipolar coupling are reduced by magic angle spinning at 54.7°. Fast spinning at the magic angle notably improves the resolution of the spectrum. Consequently, this technique is useful to acquire high-resolution NMR spectra. Previous studies showed successful results in the analysis of biological tissues, such as human liver,[20] rat liver,[21,22] mouse intestine,[23] human breast,[24] human brain,[25] and rat testicular tissues.[26] HR-MAS techniques have also been applied to cultured cells[27] such as bacteria, hepatocellular carcinoma cells and parasitic protozoa.[28]

NMR-based metabolomics has two routes for analysis: target profiling and pattern recognition (non-targeted approach or chemometric approach).[29-31] Target profiling provides a new approach to explore the specific metabolic effects of several conditions in biological systems. In this case, the compounds or metabolites are formally identified and quantified. Metabolic profiling was developed over the last decades and has been applied in various research areas including toxicology, drug development, pharmacology, foods, and nutrition. The results of target profiling were also used in disease diagnosis, *e.g.*, in the search for metabolic biomarkers for cancers. In the second method, pattern recognition is used to interpret metabolic profiles over many samples based on spectral patterns and the intensity of peaks. Pattern recognition requires software that can easily differentiate between the normal condition and disease. Chemometrics involves the use of mathematics, statistics, and computer science to obtain information in chemistry, biochemistry and chemical engineering from spectral data. When using this method, the 1D NMR spectrum is initially split up into smaller parts of bins. This process is called 'binning', and it yields isolated peaks and specific features. The binning data are analyzed using a multivariate statistical analysis. The most common form is principal component analysis (PCA),[32] which can identify how one sample is different from the others. PCA can determine the variables that contribute most to this difference and whether these variables contribute in the same way or independently from each other. PCA methods can use visual or graphical grouping, and can also be extended to higher-order analysis. Not only chemometric or statistical approaches but also spectral analysis is used in metabolomics. In this study, SIMCA-P+ software[33] was used for multivariate analysis. SIMCA-P+ is used for the analysis of PCA, and PLS-DA (partial least square-discriminant analysis) and OPLS-DA (orthogonal partial least squares-discriminant analysis).[34,35] Currently, OPLS-DA is also a popular method that has higher discrimination than PCA. In addition, OPLS-DA leads to improved interpretation.

The most common human metabolites detected by metabolomics studies are lipids (saturated and unsaturated), glucose, taurine, lactate, fumarate, malate, cholesterol, choline-containing compounds (*e.g.*, choline, phosphochline, phosphatidylcholine, glycerophosphocholine, and phosphoethanolamne), and amino acids. In previous reports, human

colorectal cancer was related to several biomarkers, such as butyrate, acetate, lipids (saturated and unsaturated), choline-containing compounds, and amino acids (*e.g.*, leucine, proline, cysteine).[2] The presence of these biomarkers varies depending on the type and species of the sample. For instance, Monleón *et al.*[47] showed that in human fecal water extracts, normal and cancer samples showed different contents of butyrate, acetate, leucine, proline, and cysteine as determined by NMR spectroscopy. Chan *et al.*[2] showed that human tissue samples contained lipids, choline-containing compounds, taurine, scyllo-inositol, glycine, polyethylene glycol, phosphoethanolamine, phosphocholine, lactate, and glucose using HR-MAS NMR analysis. In the present study, we investigated the differences between normal and cancerous colorectal tissue using HR-MAS NMR spectroscopy with two different analysis methods; target profiling and multivariate analysis techniques.

Experimental Section

Sample Collection and Preparation. Nine tissue samples from human colon cancer patients were obtained from Jeju National University Hospital (jeju, Korea). This study was approved by the institutional review board for ethics of Jeju National University Hospital (IRB: 2011-38) and by informed written consent from patients. After the biopsy, the tissue was divided into normal and cancer samples and then immediately frozen and stored at −80 °C until measurement. Each sample was weighed to 20 mg immediately prior to NMR experiment. A zirconium rotor (4-mm O.D.) was used to prepare slices of the specimens. The total volume was adjusted to 45 μL with deuterium oxide to provide field lock, and the samples also contained 2 mM TSP-d_4 (3-(trimethylsilyl) propanoic-2,2,3,3-d_4 acid sodium salt) as a reference.[2,27,36] The rotor was capped by its lid and marked using black ink for monitoring of the spinning speed. Each sample was prepared as soon as possible before NMR measurement to prevent contamination or enzymatic decomposition.

NMR Spectroscopy. All spectra were acquired at 11.7 T (500MHz) using a Varian INOVA spectrometer operating at ¹H frequency and equipped with a 4-mm gHX nanoprobe. High-resolution ¹H NMR metabolic profiling of intact biopsy tissue samples was achieved using magic angle spinning (MAS) at 54.7° with respect to the direction of the magnetic field. All data were collected at a spinning rate of 2,000 Hz and the spectra were checked between the water peak and the sideband, which coincide with the spin rate. The single-pulse 1D water suppression experiment was performed on all the samples. Deuterium oxide provided field frequency lock, and TSP-d_4 was used as an internal chemical shift reference. The acquisition time, pre-saturation delay, and relaxation delay time were 2 s, 0.02 s, and 1s, respectively. In total, 512 scans were collected at a spectral width of 8000 Hz, and the temperature was set at 298 K. All data were Fourier-transformed and calibrated to TSP-d_4 as 0.00 ppm using Chenomx NMR suite 6.0 professional.

Data Analysis. All spectra were processed and assigned by Chenomx NMR suite 6.0 professional (Chenomx Inc., Edmonton, Canada) and the Chenomx 500 MHz library database which is included 328 metabolites. Chenomx NMR suite is integrated sets of tools allow to identify and quantify metabolites in NMR spectra. The Chenomx reference libraries contain hundreds of fully searchable pH compounds models. All data were converted to the frequency domain and corrected for phase and baseline, and then the TSP-d_4 singlet peak was adjusted to 0.00 ppm. The target profiling method required confirmation of changes of a specific metabolite, followed by a comparison of data from the normal and cancer samples using Chenomx. The pattern recognition method required statistical analysis software, and in this study the SIMCA-P+ 12.0 software package (Umetrics, Umea, Sweden) was utilized to identify differences in metabolic profiles between normal and cancer data. The spectra were normalized to the total area and then binned with 0.01 ppm using Chenomx. The water region from 0.5 ppm to 4.5 ppm and reference peak were excluded prior to analysis. The OPLS-DA analysis was performed to differentiate between the cancer and normal groups.
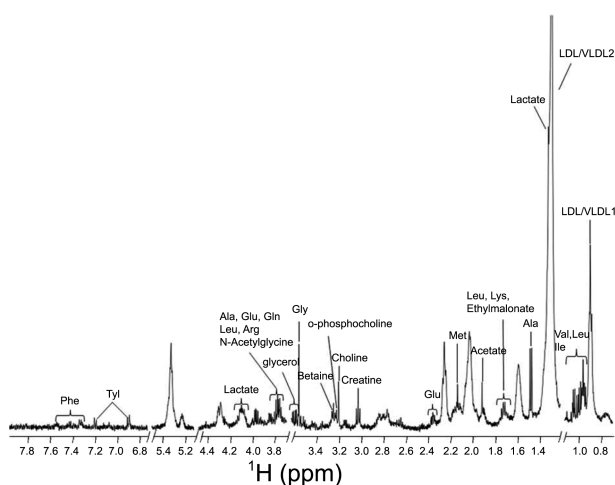
Results and Discussion

NMR Spectral Assignments. All ^{1}H NMR spectra were Fourier-transformed, and phase and baseline were corrected manually using MestreNova Suite 5.3.1 (Mestrelab Research, USA). The spectra were referenced and quantified with the TSP-d_4 peak as 0.00 ppm and 2 mM. In the normalization process, the TSP-d_4 (0.0-0.49 ppm) and water (4.5-4.7 ppm) regions were removed prior to analysis using MestreNova. 1D NMR spectra of cancer and normal tissues obtained from 9 patients with colorectal cancer are presented in Figure 1. The spectra show metabolites similar to those in previous reports, with strong peaks from 0.8 to 4.5 ppm. The major metabolites identified in the spec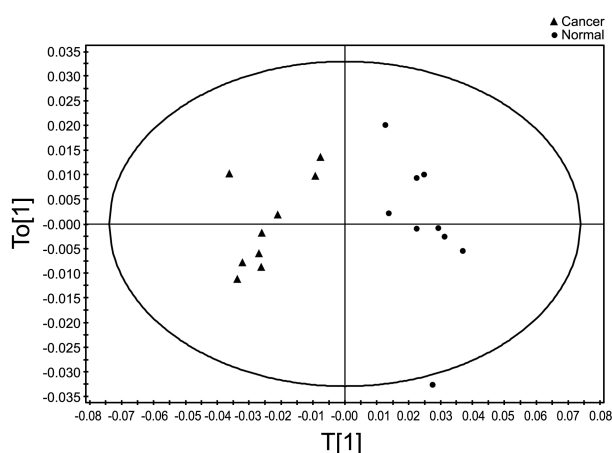tra included acetate, lactate, ethylmalonate, choline, *o*-phosphocholine, myo-inositol, creatine, taurine, lipid components, several amino acids, and sugars and these are summarized in Table 1. These metabolites were identified using the Chenomx 500 MHz library

Table 1. ^{1}H chemical shift of metabolites found in HR-MAS spectra of human colon tissues. Abbreviation: LDL/VLDL, low-density lipoprotein/very low-density lipoprotein. Peak multiplicities: s, singlet; d, doublet; t, triplet; dd, doublet of doublet; q, quartet; and m, multiplet

Metabolite	δ ^{1}H (multiplicity)
LDL1/VLDL1	0.90 (m)
Ethylmalonate	0.87 (t), 1.71 (p), 3.00 (t)
2-Hydroxybutyrate	0.89 (t), 1.64 (m), 1.73 (m), 3.99 (m)
Isoleucine	0.93 (t), 1.00 (d), 1.25 (m), 1.46 (m), 1.97 (m), 3.66 (d)
Leucine	0.94 (d), 0.96 (d), 1.70 (m), 3.73 (m)
Valine	0.98 (d), 1.03 (d), 2.26 (m), 3.60 (d)
LDL2/VLDL2	1.29 (m)
Lactate	1.31 (d), 4.11 (q)
Alanine	1.47 (d), 3.78 (q)
Acetate	1.90 (s)
Arginine	1.64 (m), 1.72 (m), 1.90 (m), 3.24 (t), 3.76 (t)
Lysine	1.42 (m), 1.50 (m), 1.72 (m), 1.87 (m), 1.91 (m), 3.02 (t), 3.75 (t)
Proline	1.98 (m), 2.03 (m), 2.06 (m), 2.34 (m), 3.32 (m), 3.41 (m), 4.12 (m)
N-Acetylglutamine	1.91 (m), 2.02 (s), 2.10 (m), 2.31 (t), 2.33 (t), 4.15 (m), 7.94 (d)
N-Acetylglycine	2.03 (s), 3.74 (d), 7.98 (s)
Methionine	2.11 (m), 2.12 (s), 2.19 (m), 2.63 (t), 3.85 (m)
Glutamate	2.04 (m), 2.12 (m), 2.32 (t), 2.36 (t), 3.75 (m)
Glutamine	2.11 (m), 2.14 (m), 2.42 (m), 2.46 (m), 3.77 (t)
Glutathione	2.14 (m), 2.17 (m), 2.52 (m), 2.56 (m), 2.93 (dd), 2.97 (dd), 3.78 (m), 4.56 (m)
Acetoacetate	2.25 (s), 3.42 (s)
Pyruvate	2.34 (s)
Aspartate	2.67 (dd), 2.80 (dd), 3.89 (dd)
Asparagine	2.85 (m), 2.95 (m), 4.00 (dd),
Creatine	3.01 (s), 3.92 (s)
Cysteine	3.03 (m), 3.09 (m), 3.98 (dd)
Malonate	3.12 (s)
Choline	3.19 (s), 3.50 (m), 4.06 (m)
o-Phosphocholine	3.21 (s), 3.58 (m), 4.16 (m)
Taurine	3.25 (t), 3.42 (t)
Carnitine	2.41 (m), 2.45 (m), 3.21 (s), 3.39 (m)
Betaine	3.24 (s), 3.89 (s)
myo-Inositol	3.27 (t), 3.53 (dd), 3.62 (t), 4.05 (t)
Glycine	3.55 (s)
Glycerol	3.56 (m), 3.65 (m), 3.78 (m)
Galactarate	3.96 (s), 4.26 (s)
Tyrosine	3.04 (m), 3.19 (m), 3.93 (m), 6.89 (d), 7.18 (d)
Phenylalanine	3.12 (m), 3.28 (m), 3.99 (m), 7.32 (d), 7.37 (m), 7.42 (m)
Uracil	5.79 (d), 7.54 (d)
Hypoxanthine	8.18 (s), 8.20 (s)

Figure 1. Normal tissue sample was selected as an example for detailed signal assignment with TSP-d_4 (δ 0.00 ppm). The following metabolites were identified using the Chenomx 500 MHz library (the ethanol signal was eliminated).
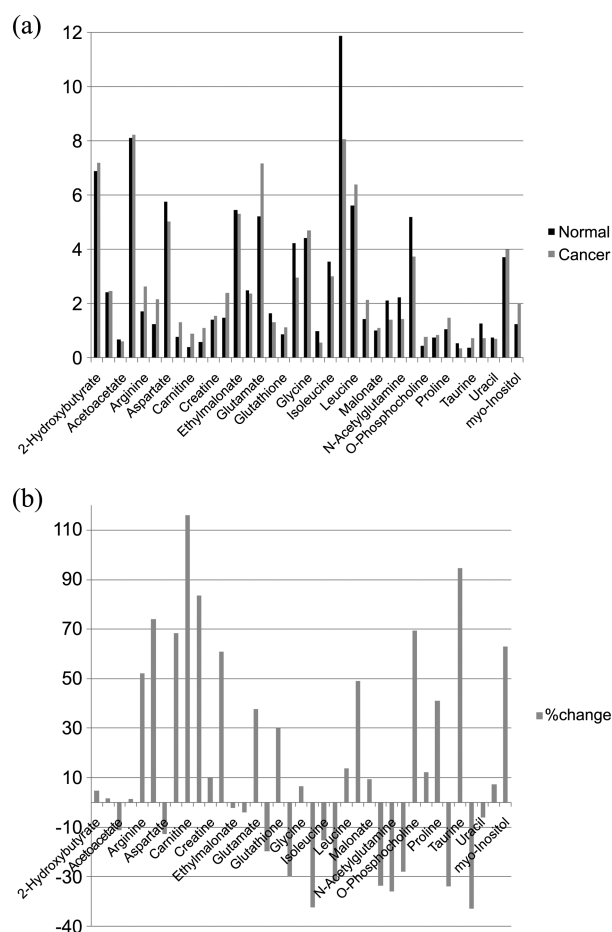
Figure 2. OPLS-DA score plot (comp. 1 *vs* comp. 2) obtained from the NMR spectra of 18 tissue samples using SIMCA-P+ 12.0. 18 samples were classified into two groups. ▲: cancer ●: normal.

Figure 3. (a) Relative concentration of major metabolites represented in the graph. The concentrations of metabolites were calculated from the integration of peak areas using Chenomx. (b) Percent change of colorectal cancer tissues metabolite levels relative to normal tissues. % Change = ([cancer] – [normal])/ [normal] × 100.

database and the literature.[2,17-20] Most metabolites for colorectal cancer were identified. The level of many metabolites was similar for normal and cancer samples. However, the normal tissue samples had higher levels of LDL1/VLDL1, LDL2/VLDL2 and lactate than cancer tissue samples, whereas cancer tissue samples had higher levels of taurine, myo-inositol and arginine. These findings are similar to those of previous studies that analyzed the metabolites of colorectal cancer using GC/MS and HR-MAS NMR.[2]

Multivariate Analysis. Nine cancer and normal samples from the same colorectal cancer patients were analyzed by ^{1}H NMR. To achieve multivariate analysis, the regions corresponding to the water solvent and TSP-d_4 were excluded, and the remaining spectra in the region of 0.5-4.5 ppm were divided into 0.01-ppm bins. The OPLS-DA analysis was conducting using the centering scale (Ctr scale) with SIMCA-P+ 12.0. The OPLS-DA score plot was used to determine whether the metabolic fingerprints of colorectal tissues were sufficiently unique to identify metabolic markers for cancer. Figure 2 shows the OPLS-DA score plot for the first two principal components from the present spectra of the two groups, namely, normal and cancer. OPLS-DA was applied for the separation of the two groups, where Hotelling's T region with a 95% confidence interval was used for the modeled variation. The values of R^2X, R^2Y were 0.978 and 0.891, respectively, and Q^2, which describes the predictability of the model, was −0.0437. The score plot shows clearly clustered groups for the normal and cancer samples except for one of cancer sample.

Differences of Metabolites Between Normal and Cancer Tissues. Figure 3 presents the relative concentration of metabolites in normal and colorectal cancer tissues. The concentrations of some amino acids (*i.e.*, Arg, Asp, Cys, Glu, Leu, Lys, and Pro), betaine, taurine, myo-inositol, choline, *o*-phosphocholine, carnitine, and glutathione were higher in cancer tissue. In contrast, the concentrations of glycerol, lactate, *N*-acetylglutamine, *N*-acetylglycine, and lipids were relatively higher in normal tissues.

Arginine gave multiplets at 1.65, 1.73, 1.90, and 1.92 ppm, and triplets 3.25 and 3.78 ppm. Arginine plays a role in the energy metabolism of muscle and is also a final intermediate in the urea cycle before the formation of urea *via* arginase.[37] Taurine is broadly distributed in animal tissue and involved in biological roles such as antioxidation, osmoregulation, membrane stabilization, and bile salt formation.[38-40] Increased levels of taurine in cancer may be related to a more active metabolism and stimulation of glycolysis, as there is a high energetic demand from cancer cells.[41] Myo-inositol is an important metabolite in regulating vital cellular functions such as signal transduction, proliferation, and differentiation.[42] Myo-inositol is also a component of cell membranes and an essential nutrient required by human cells for growth and survival. Carnitine is formed from amino acids such as lysine and methionine in the liver and kidney.[43] In this study, carnitine levels were increased in the cancer samples, which may indicate reduced methionine. Glutathione is an antioxidant that prevents damage to important cellular components caused by reactive oxidative species such as free radicals.[44-46] Our HR-MAS NMR analysis showed an increased level of glutathione in cancer tissues, which

suggests activation of the glutathione synthesis pathway. Choline-containing compounds are major components of the cell membrane. Our NMR results showed higher levels of choline and *o*-phosphocholine in cancer tissues relative to normal tissues. Generally, in mammalian tumors, activation of the CDP-choline pathway and increased choline and phosphocholine levels are typically observed. This result is consistent with several previous studies *in vitro* and *in vivo*.[48]

Glycerol levels were found to lower in normal tissue in our analysis, which suggests that the consumption and degradation of glycerol is decreased in cancer tissues. However, glycerol was previously shown to increase in cancer tissue in earlier studies.[49]

Lactate gave a doublet at 1.32 ppm and a quadruplet at 4.11 ppm, which is an end product of glycolysis that increases rapidly during hypoxia and ischemia. Increased lactate levels have been associated with various cancer.[50] In this study, this result was unexpected and should be addressed with further research.

Conclusions

In conclusion, our HR-MAS NMR-based metabolomics technique showed significant differences between normal and colorectal cancer in intact tissues without requiring any extraction process to obtain a 1D proton NMR spectrum. Our result may provide a good diagnostic method and biomarker for colorectal cancer.

References

1. Ma, Y. L.; Qin, H. L.; Liu, W. J.; Peng, J. Y.; Huang, L.; Zhao, X. P.; Cheng, Y. Y. *Digestive Diseases and Sciences* **2009**, *54*, 2655-2662.
2. Chan, E. C.; Koh, P. K.; Mal, M.; Cheah, P. Y.; Eu, K. W.; Backshall, A.; Cavill, R.; Nicholson, J. K.; Keun, H. C. *Journal of Proteome Research* **2009**, *8*, 352-361.
3. Nicholson, J. K.; Connelly, J.; Lindon, J. C.; Holmes, E. *Nature Reviews Drug Discovery* **2002**, *1*, 153-161.
4. Nicholson, J. K.; Lindon, J. C. *Nature* **2008**, *455*, 1054.
5. Nicholson, J. K.; Lindon, J. C.; Holmes, E. *Xenobiotica* **1999**, *29*, 1181-1189.
6. Bezabeh, T.; Somorjai, R. L.; Smith, I. C. *Magnetic Resonance in Chemistry* **2009**, *47*, S54-S61.
7. Cheng, L. L.; Lean, C. L.; Bogdanova, A.; Wright, S. C., Jr.; Ackerman, J. L.; Brady, T. J.; Garrido, L. *Magnetic Resonance in Medicine* **1996**, *36*, 653-658.
8. Rocha, C. M.; Carrola, J.; Barros, A. S.; Gil, A. M.; Goodfellow, B. J.; Carreira, I. M.; Bernardo, J.; Gomes, A.; Sousa, V.; Carvalho, L.; Duarte, I. F. *Journal of Proteome Research* **2011**, *10*, 4314-4324.
9. Holmes, E.; Loo, R. L.; Stamler, J.; Bictash, M.; Yap, I. K.; Chan, Q.; Ebbels, T.; De Iorio, M.; Brown, I. J.; Veselkov, K. A.; Daviglus, M. L.; Kesteloot, H.; Ueshima, H.; Zhao, L.; Nicholson, J. K.; Elliott, P. *Nature* **2008**, *453*, 396-401.
10. Carrola, J.; Rocha, C. M.; Barros, A. S.; Gil, A. M.; Goodfellow, B. J.; Carreira, I. M.; Bernardo, J.; Gomes, A.; Sousa, V.; Carvalho, L.; Duarte, I. F. *Journal of Proteome Research* **2011**, *10*, 221-230.
11. Zhang, S.; Nagana Gowda, G. A.; Asiago, V.; Shanaiah, N.; Barbas, C.; Raftery, D. *Analytical Biochemistry* **2008**, *383*, 76-84.
12. OuYang, D.; Xu, J.; Huang, H.; Chen, Z. *Applied Biochemistry and Biotechnology* **2011**, *165*, 148-154.
13. MacIntyre, D. A.; Jiménez, B.; Lewintre, E. J.; Martin, C. R.; Schäfer, H.; Ballesteros, C. G.; Mayans, J. R.; Spraul, M.; García-Conde, J.; Pineda-Lucena, A. *Leukemia* **2010**, *24*, 788-797.
14. Tiziani, S.; Lopes, V.; Günther, U. L. *Neoplasia* **2009**, *11*, 269-276.
15. Blasco, H.; Corcia, P.; Moreau, C.; Veau, S.; Fournier, C.; Vourc'h, P.; Emond, P.; Pradat, P. F.; Praline, J.; Devos, D.; Nadal-Desbarats, L.; Andres, C. R. *PLoS One* **2010**, *5*, e13223.
16. Rosenling, T.; Stoop, M. P.; Smolinska, A.; Muilwijk, B.; Coulier, L.; Shi, S.; Dane, A.; Christin, C.; Suits, F.; Horvatovich, P. L.; Wijimenga, S. S.; Buydens, L. M.; Vreeken, R.; Hankemeier, T.; van Gool, A. J.; Luider, T. M.; Bischoff, R. *Clinical Chemistry* **2011**, *57*, 1703-1711.
17. Beckonert, O.; Coen, M.; Keun, H. C.; Wang, Y.; Ebbels, T. M.; Holmes, E.; Lindon, J. C.; Nicholson, J. K. *Nature Protocols* **2010**, *5*, 1019-1032.
18. Moka, D.; Vorreuther, R.; Schicha, H.; Spraul, M.; Humpfer, E.; Lipinski, M.; Foxall, P. J. D.; Nicholson, J. K.; Lindon, J. K. *Analytical Communications* **1997**, *34*, 107-109.
19. Rooney, O. M.; Troke, J.; Nicholoson, J. K.; Griffin, J. L. *Magnetic Resonance in Medicine* **2003**, *50*, 925-930.
20. Gao, H.; Lu, Q.; Liu, X.; Cong, H.; Zhao, L.; Wang, H.; Lin, D. *Cancer Science* **2009**, *100*, 782-785.
21. Hong, Y. S.; Coen, M.; Rhode, C. M.; Reily, M. D.; Robertson, D. G.; Holmes, E.; Lindon, J. C.; Nicholson, J. K. *Magnetic Resonance in Chemistry* **2009**, *47*, S47-S53.
22. Bollard, M. E.; Garrod, S.; Holmes, E.; Lindon, J. C.; Humpfer, E.; Spraul, M.; Nicholson, J. K. *Magnetic Resonance in Medicine* **2000**, *44*, 201-207.
23. Backshall, A.; Alferez, D.; Teichert, F.; Wilson, I. D.; Wilkinson, R. W.; Goodlad, R. A.; Keun, H. C. *Journal of Proteome Research* **2009**, *8*, 1423-1430.
24. Cheng, L. L.; Chang, I. W.; Smith, B. L.; Gonzalez, R. G. *Journal of Magnetic Resonance* **1998**, *135*, 194-202.
25. Cheng, L. L.; Chang, I. W.; Louis, D. N.; Gonzalez, R. G. *Cancer Research* **1998**, *58*, 1825-1832.
26. Griffin, J. L.; Troke, J.; Walker, L. A.; Shore, R. F.; Lindon, J. C.; Nicholson, J. K. *FEBS Letters* **2000**, *486*, 225-229.
27. Kim, S.; Kim, S.; Lee, S.; Kwon, B.; Choi, J.; Hyun, J. W.; Kim, S. *Bulletin of the Korean Chemical Society* **2011**, *32*, 2021-2026.
28. Moreno, B.; Rodrigues, C. O.; Bailey, B. N.; Urbina, J. A.; Moreno, S. N.; Docampo, R.; Oldfield, E. *FEBS Letters* **2002**, *523*, 207-212.
29. Shulaev, V. *Briefings in Bioinformatics* **2006**, *7*, 128-139.
30. Wishart, D. S. *Briefings in Bioinformatics* **2007**, *8*, 279-293.
31. Wishart, D. S. *Trends in Analytical Chemistry* **2008**, *27*, 228-237.
32. Wold, S.; Esbensen, K.; Geladi, P. *Chemometrics and Intelligent Laboratory Systems* **1987**, *2*, 37-52.
33. Wold, S. *Pattern Recognition* **1976**, *8*, 127-139.
34. Trygg, J.; Wold, S. *Journal of Chemometrics* **2002**, *16*, 119-128.
35. Bylesjö, M.; Rantalainen, M.; Cloarec, O.; Nicholson, J. K.; Holmes, E.; Trygg, J. *Journal of Chemometrics* **2006**, *20*, 341-351.
36. Waters, N. J.; Garrod, S.; Farrant, R. D.; Haselden, J. N.; Connor, S. C.; Connelly, J.; Lindon, J. C.; Holmes, E.; Nicholson, J. K. *Analytical Biochemistry* **2000**, *282*, 16-23.
37. Tikunov, A. P.; Johnson, C. B.; Lee, H.; Stoskopf, M. K.; Macdonald, J. M. *Marine Drugs* **2010**, *8*, 2578-2596.

38. Aruoma, O. I.; Halliwell, B.; Hoey, B. M.; Butler, J. *Biochemical Journal* **1988**, *256*, 251-255.
39. Schaffer, S.; Takahashi, K.; Azuma, J. *Amino Acids* **2000**, *19*, 527-546.
40. Birdsall, T. C. *Alternative Medicine Review* **1998**, *3*, 128-136.
41. Kirk, K.; Kirk, J. *FEBS Letters* **1993**, *336*, 153-158.
42. Downes, C. P.; Macphee, C. H. *European Journal of Biochemistry* **1990**, *193*, 1-18.
43. Krajcovicová-Kudláčková, M.; Simoncic, R.; Béderová, A.; Babinská, K.; Béder, I. *Physiological Research* **2000**, *49*, 399-402.

44. Halliwell, B.; Gutteridge, J. *Free Radicals in Biology and Medicine* **1999**, 3rd ed. (Oxford Univ. Press, Oxford).
45. Meister, A. *Science* **1983**, *220*, 472-477.
46. Martindale, J. L.; Holbrook, N. J. *Journal of Cellular Physiology* **2002**, *192*, 1-15.
47. Monleón, D.; Morales, J. M.; Barrasa, A.; López, J. A.; Vázquez, C.; Celda, B. *NMR in Biomedicine* **2009**, *22*, 342-348.
48. Nakagami, K.; Uchida, T.; Ohwada, S.; Koibuchi, Y.; Suda, Y.; Sekine, T.; Morishida, Y. *Cancer Science* **1999**, *90*, 419-424.
49. Lin, E. C. *Annual Review of Biochemistry* **1977**, *46*, 765-795.
50. Waterhouse, C. *Cancer* **1974**, *33*, 66-71.