DOI:10.1021/jf102194t

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Discrimination of the Geographical Origin of Beef by ^{1}H NMR-Based Metabolomics

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The geographical origin of beef is of increasing interest to consumers and producers due to "mad cow" disease and the implementation of the Free Trade Agreement (FTA). In this study, ^{1}H NMR spectroscopy coupled with multivariate statistical analyses was used to differentiate the geographical origin of beef samples. Principal component analysis (PCA) and orthogonal projection to latent structure−discriminant analysis (OPLS-DA) showed significant separation between extracts of beef originating from four countries: Australia, Korea, New Zealand, and the United States. The major metabolites responsible for differentiation in OPLS-DA loading plots were succinate and various amino acids including isoleucine, leucine, methionine, tyrosine, and valine. A one-way ANOVA was performed to statistically certify the difference in metabolite levels. The data suggest that NMR-based metabolomics is an efficient method to distinguish fingerprinting difference between raw beef samples, and several metabolites including various amino acids and succinate can be possible biomarkers for discriminating the geographical origin of beef.

KEYWORDS: Beef; geographical origin; NMR; metabolomics; chemometric analysis

INTRODUCTION

Food quality and safety are common concerns of human society and the international community. As a result of the Free Trade Agreement (FTA), a global beef industry has emerged. Furthermore, concerns over "mad cow" disease have increased awareness of food safety. Many consumers now demand objective and authentic information about food quality and origins. These demands call for, among other tools, an accurate and reliable analytical method to determine the geographical origin of beef. Many meat products have an added value if they are produced in particular regions. The development of appropriate tools would help prevent deliberate or accidental mislabeling of origin (*1*−*5*).

The quality or origin of food is mostly determined by its biochemical composition, and this biochemical (i.e., metabolite) profile is an important factor in determining food quality and origin. Recently, NMR spectroscopy coupled with multivariate analysis has been applied to obtain metabolite profiles of various kinds of food including salmon (*6*, *7*), meat (*1*, *8*), honey (*9*−*11*), milk (*12*), olive oil (*13*), wine (*14*, *15*), and other plants (*16*−*19*). The metabolite content of a given sample constitutes a unique fingerprint for that product. Most metabolomic studies have taken advantage of a multivariate statistical approach to evaluate large sets of information obtained by advanced analytical techniques and to discriminate redundant information (*18*, *20*, *21*). This nontargeted metabolite profiling can rapidly visualize differences of metabolite patterns among foods from different origins. On the other hand, targeted profiling makes it possible to identify and quantify metabolites at low concentration and in overlapping spectral regions. Especially, this approach has a significant advantage in discrimination based on quantitative differences of their metabolites in food. Therefore, such quantitative metabolite profiling can be used to effectively discover potential biomarkers useful in identifying and authenticating the metabolic specifications of a particular food (*17*, *22*).

Among the possible analytical techniques, NMR spectroscopy provides detailed information about specific components of complex mixtures. The use of NMR also simplifies sample preparation and decreases the time required for analysis. ^{1}H NMR spectroscopy is therefore a particularly powerful analytical technique for biomarker characterization (*6*, *15*). Because NMR analyses produce highly complex sets of data, multivariate or pattern recognition techniques such as principal component analysis (PCA) or orthogonal projections to latent structures−discriminant analysis (OPLS-DA) have been designed specifically to analyze NMR-derived data.

Determination of the geographical origin of beef has been mainly studied using stable isotope ratio analyses (*23*−*25*). The NMR technique, in the past few years, has been proposed as a potential tool for determining food quality and geographical origin. In complex matrices such as foods, several chemical compounds

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 Published on Web 09/10/2010

Table 1. Beef Samples Used in This Study

country	part	no. of samples
Australia	chuck	10
Korea	sirloin	10
New Zealand	sirloin	10
United States	chuck	10

can be rapidly observed at the same time and quantified reproducibly (*26*, *27*). An NMR-based metabolomic approach to determine origin was applied to dried beef samples by HR-MAS NMR spectroscopy (*8*). However, no metabolite profiles of the origins of raw beef have been reported to date. In this study, ^{1}H NMR spectroscopy, followed by multivariate analysis techniques, was applied to metabolomic analyses of beef extract samples obtained from four countries (Australia, Korea, New Zealand, and the United States). Metabolite profiling was applied to investigate the metabolite differences of raw beef from different origins and to identify the significant metabolites. The typical metabolite profile of raw beef can be correlated with the environmental parameters of its geographical origin (*28*, *29*), feeding regimen (*30*, *31*), breed (*31*), and production system (*29*, *32*). In addition, slaughter processing, post-mortem aging, sex of the animals, final age, and weight at slaughter are also important factors that can influence the biochemical components of beef (*32*−*34*). The environmental conditions of a given area impart specific characteristics to the product and are a primary factors determining typicality (*35*). The chemical or metabolite composition of beef can also differ in accordance with the different breeds of cattle, feeding regimens (grain-fed or pasture-fed cattle), the production system (extensive pasture or intensive feedlot systems), difference during the preslaughter phase, and the postslaughter environment of each country. The present study aimed to integrate the metabolite profiling data in the raw beef obtained from different geographic areas and identify potential marker candidates for determining the geographical origin of beef.

MATERIALS AND METHODS

Sample Preparation. Forty authentic raw beef samples were collected from four countries (Australia, Korea, New Zealand, and the United States) (**Table 1**). All imported beef samples were collected before customs entry by the Central Customs Laboratory and Scientific Service; the Korean beef samples were collected from Korean slaughterhouses. Different countries have different cuts of beef and different names for these cuts. Therefore, the sirloin of Korean and New Zealand beef and the chuck of Australian and U.S. beef were selected for analysis. All frozen samples were stored at −80 °C until required for NMR analysis. From each sample, about 200 mg of beef was put into a 1.5 mL tube containing 2.8 mm zirconium oxide beads and homogenized twice at 5000 rpm with 350 μL of methanol (d_4) and 150 μL of 0.2 M (pH 7) sodium phosphate buffer for 20 s using a Precellys 24 tissue grinder (Bertin Technologies, Ampère Montigny-le-Bretonneux, France). After homogenization, 210 μL of methanol (d_4), 90 μL of 0.2 M (pH 7) sodium phosphate buffer, and 400 μL of chloroform were added to the tube. This mixture was vortexed vigorously for 1 min. The samples were allowed to separate for 15 min and centrifuged at 13000 rpm for 10 min at 4 °C. The upper layer was transferred in 630 μL aliquots to new 1.5 mL Eppendorf tubes and mixed with 0.25 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate (70 μL; DSS, 97%) dissolved in deuterium oxide (D_2O, 99.9%). The mixture was then centrifuged at 13000 rpm for 5 min. The supernatants (600 μL) were transferred into 5 mm NMR tubes.

NMR Spectroscopy. ^{1}H NMR spectra were acquired on a VNMRS 600-MHz NMR using a triple-resonance HCN salt-tolerant cold probe (Varian Inc., Palo Alto, CA). A NOESY-PRESAT pulse sequence was applied to suppress the residual water signal. D_2O and DSS provided a field frequency lock and chemical shift reference (^{1}H, δ 0.00), respectively. For each sample, 64 transients were collected into 32K data points using a spectral width of 9615.4 Hz with a relaxation delay of 2.0 s, an acquisition time of 4.00 s, and a mixing time of 100 ms. A 0.5 Hz line-broadening function was applied to all spectra prior to Fourier transformation (FT). Assignments of NMR signals were based on total correlation spectroscopy [two-dimensional (2D) ^{1}H−^{1}H TOCSY], heteronuclear multiple bond correlations (2D ^{1}H−^{13}C HMBC), heteronuclear single quantum correlations (2D ^{1}H−^{13}C HSQC), spiking experiments, and comparisons to the literature (*8*, *36*). Additional 2D NMR experiments were performed using a Bruker Biospin Avance 800 MHz NMR spectrometer.

NMR Data Preprocessing and Multivariate Statistical Analysis. All NMR spectra were phased and baseline corrected by Chenomx NMR suite version 6.0 (Chenomx Inc., Edmonton, AB, Canada). The regions corresponding to the solvent and DSS (4.75−5.12, 3.30−3.33, and 0.0−0.7 ppm) were excluded, and the remaining spectral regions were divided into 0.01 ppm bins. The spectra were then normalized to the total spectral area and converted to ASCII format. The ASCII format files were imported into MATLAB (R2006a; Mathworks, Inc., Natick, MA), and all spectra were aligned using the correlation optimized warping (COW) method (*37*). The resultant data sets were then imported into SIMCA-P version 12.0 (Umetrics, Umeå, Sweden) for chemometric analyses. All imported data were Pareto scaled for multivariate analysis. Pareto scaling, in which each variable is divided by the square root of the standard deviation, gives greater weight to the NMR data variables with larger intensity but is not as extreme as using unscaled data. It is usually used when there is a very large dynamic range in the data set (*38*, *39*). Principal component analysis (PCA) was initially performed to examine the intrinsic variation in the data set and obtain an overview of variation among the groups. Orthogonal projections to latent structures−discriminant analysis (OPLS-DA) was employed to maximize the separation among the groups. The OPLS model maximizes the covariance between the measured data of X variable (peak intensities in NMR spectra) and the response of Y variable (classification components) within the groups (*40*−*42*). The quality of the models was described by R^2 and Q^2 values. R^2 is defined as the proportion of variance in the data explained by the models and indicates the goodness of fit. Q^2 is defined as the proportion of variance in the data predictable by the model and indicates predictability (*43*). The S-Plot was generated from OPLS-DA model to screen the metabolites contributing to the separation between groups. Two vectors, p and p(corr), were combined in a plot. p represents the covariance, and p(corr) gives the correlation for variables with respect to the component (*40*, *42*−*44*). In addition, we performed permutation tests and external validations to test the validity of the OPLS-DA models (*43*, *45*, *46*).

Targeted Metabolite Profiling. Metabolites were identified using Chenomx Profiler, a module of the Chenomx NMR Suite version 6.0. All standard NMR spectra used for metabolite identification are commercially available (Chenomx Inc.). The identities of metabolites deemed important in chemometric analyses were confirmed using 2D NMR HSQC, HMBC, and TOCSY experiments and spiking experiments. Quantification was achieved using the 600 MHz library from Chenomx NMR Suite version 6.0, which uses the concentration of a known reference signal (in this case, DSS) to determine the concentration of individual compounds. The library is based on a database of individual metabolite spectra acquired using the NOESY-PRESAT sequence and contained 260 metabolites (*17*, *22*).

Statistical Methods. A one-way ANOVA was performed using GraphPad PRISM version 5.0 (GraphPad Software, Inc., La Jolla, CA) and SPSS version 12.0 (SPSS Inc., Chicago, IL) to test the significance of differences in metabolite levels among groups of samples of different origins. The differences were tested on a 95% probability level ($p < 0.05$). Tukey's multiple-comparison tests were performed to reveal paired differences between the means (*47*).

RESULTS AND DISCUSSION

^{1}H NMR Data of Aqueous Beef Extract. Representative one-dimensional ^{1}H NMR spectra of aqueous beef extract samples from Australia, Korea, New Zealand, and the United States are shown in **Figure 1**. The vertical scale of the aromatic region was doubled for better visibility. The main aromatic signals in these extracts were attributable to carnosine and anserine, which exhibit large variability in chemical shift due to their pH sensitivity. Differences in chemical shift were adjusted by alignment in MATLAB. Metabolite assignments were based on analyses of 2D

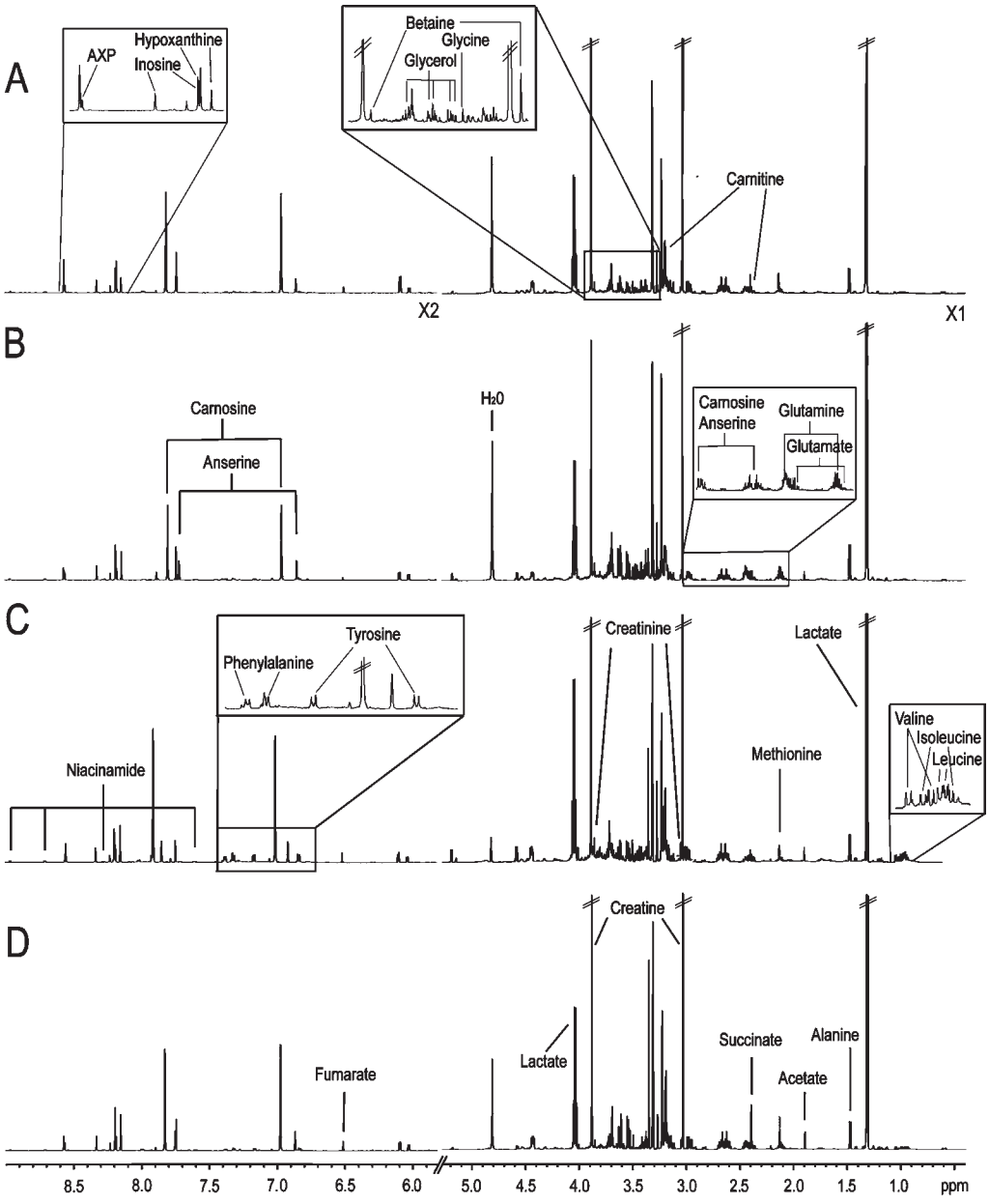

Figure 1. Representative ^{1}H NMR spectra of beef sirloin (or chuck) extracts obtained from Australia (**A**), Korea (**B**), New Zealand (**C**), and the United States (**D**). The vertical scale of the aromatic region is doubled for better visibility.

NMR using TOCSY, HMQC, HMBC and spiking experiments and information published elsewhere (*10*, *36*). TOCSY, HMQC, and HMBC experiments provided the information required to assign the most relevant compounds observed in the aqueous beef extracts. An expanded portion of the HMQC and HMBC spectra used in the assignment of metabolites is shown in **Figure 2**; the TOCSY spectra are given in the Supporting Information, Figure 1S. The ambiguous metabolites in 2D NMR analyses were identified by the addition of standard compound. Twenty-five metabolites were identified in the ^{1}H NMR spectra of beef extracts (**Table 2**). No clear visual differences were observed in the overall spectroscopic fingerprints among beef samples obtained from the four countries. However, close inspection of the spectra revealed that the chemical compositions of metabolites between beef samples obtained from different countries were distinctly different (i.e., amino acids and organic acids).

Metabolomic Analysis of Beef Extracts from Different Geographical Origins. PCA is an unsupervised classification method requiring no a priori knowledge of the data set and acts to reduce the dimensionality of multivariate data while preserving most of the variance within it (*19*). PCA and OPLS-DA score plots were used to determine whether the metabolic fingerprints of beef samples were sufficiently unique to identify metabolic markers for the different geographical regions. The PCA and OPLS-DA score plots derived from the NMR spectra of beef extracts from the four countries are given in **Figure 3**. The PCA models using projections into three dimensions show statistically significant separation among the four countries, indicating differences in metabolite composition among the beef extracts from the different countries. To maximize the separation between samples, OPLS-DA was applied. R_X^2 represents the goodness of the fit to the PCA model, and Q^2 reveals the predictability of the PCA model. The PCA model (**Figure 3A**) for distinguishing beef samples from different origins was established using seven components and revealed R_X^2 and Q^2 values of 0.741 and 0.449, respectively; the OPLS model (**Figure 3B**) was established using three predictive and one orthogonal component and revealed R_X^2, R_Y^2, and Q^2 values of 0.609, 0.848, and 0.757, respectively.

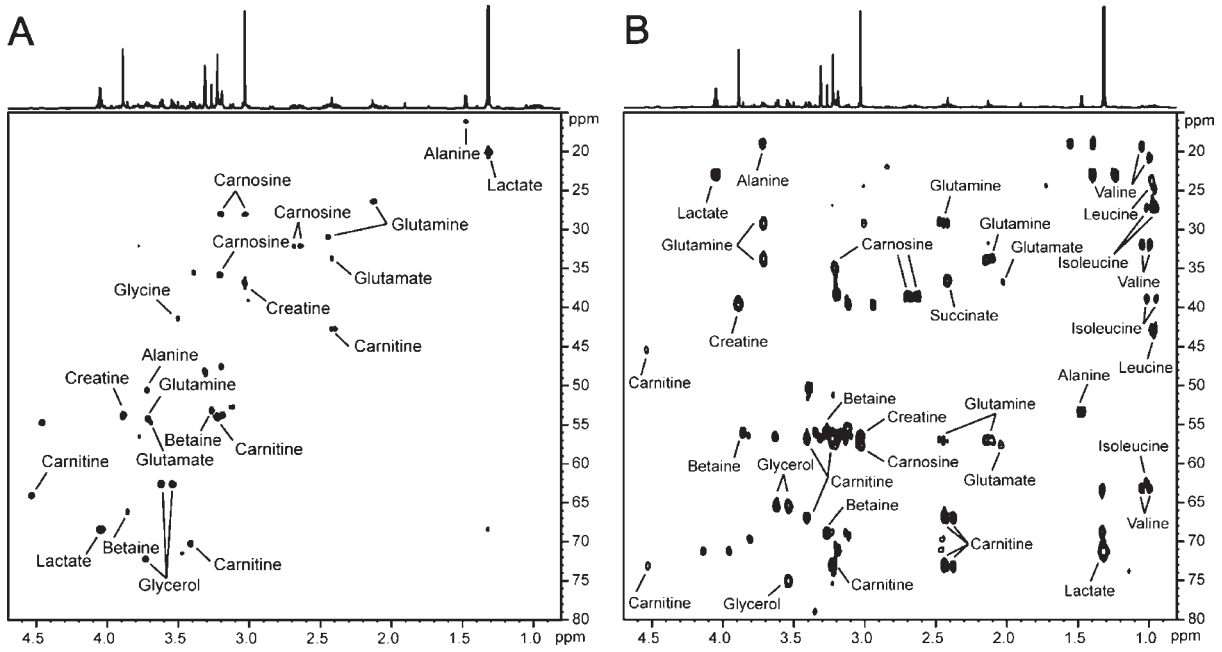

Figure 2. Representative 2D NMR spectra of beef chuck extracts obtained from the United States: (**A**) expansion of HMQC spectra; (**B**) expansion of HMBC spectra.

Table 2. Metabolites, Their Chemical Shifts (Multiplicity), and *p* Values for Pairwise Test

		p value[c]					
metabolite	chemical shift (multiplicity)[b]	AUS[d]/KOR	AUS/NZ	AUS/USA	KOR/NZ	KOR/USA	NZ/USA
acetate**[a]	1.90 (s)	<0.001	<0.001	0.001	0.977	0.993	0.904
alanine	1.48 (d), 3.81 (q)	0.861	0.399	0.774	0.853	0.998	0.923
anserine**	2.63 (m), 2.97 (dd), 3.19 (m), 3.69 (s), 4.48 (m), 6.86 (s), 7.75 (s)	0.001	<0.001	0.106	0.228	0.315	0.004
betaine*	3.27 (s), 3.85 (s)	0.923	0.105	0.914	0.324	0.582	0.024
carnitine*	2.40 (m), 3.23 (s), 3.41 (m), 4.53 (m)	0.921	0.673	0.020	0.959	0.087	0.229
carnosine**	2.65 (m), 2.97 (dd), 3.17 (m), 4.43 (m), 6.97 (s), 7.82 (s)	0.081	<0.001	0.520	0.008	0.694	<0.001
choline**	3.21 (s), 3.50 (m), 4.06 (m)	0.018	0.004	0.112	0.952	0.853	0.550
creatine**	3.03 (s), 3.88 (s)	0.180	0.038	1.000	<0.001	0.181	0.037
creatinine**	3.05 (s), 4.00 (s)	0.001	<0.001	0.194	<0.001	0.164	<0.001
fumarate*	6.51 (s)	0.339	0.348	0.898	0.009	0.748	0.100
glutamate**	2.06 (m), 2.37 (m), 3.76 (m)	0.994	<0.001	0.005	<0.001	0.01	0.167
glutamine**	2.12 (m), 2.44 (m), 3.70 (m)	0.357	0.001	0.992	0.065	0.519	0.002
glycerol**	3.54 (dd), 3.62 (dd), 3.72 (m)	<0.001	0.002	<0.001	0.805	0.331	0.060
glycine**	3.50 (s)	0.992	<0.001	0.820	<0.001	0.936	0.002
hypoxanthine**	8.15 (s), 8.19 (s)	0.003	<0.001	<0.001	0.056	0.880	0.246
inosine	3.83 (m), 3.91 (m), 4.22 (m), 4.38 (m), 4.69 (m), 6.03 (d), 8.19 (s), 8.33 (s)	0.073	0.252	0.685	0.919	0.500	0.865
isoleucine**	0.95 (t), 1.02 (d), 1.25 (m), 1.47 (m), 1.93 (m), 3.68 (d)	0.111	<0.001	0.428	<0.001	0.858	<0.001
lactate**	1.32 (d), 4.03 (q)	0.085	<0.001	0.236	0.001	0.951	<0.001
leucine**	0.96 (d), 0.98 (d), 1.63 (m), 1.69 (m), 1.96 (m), 3.72 (t)	0.212	<0.001	0.392	<0.001	0.980	<0.001
methionine**	2.13 (s), 2.14 (m), 2.66 (dd), 3.78 (m)	0.830	0.002	0.888	<0.001	0.999	<0.001
niacinamide**	7.60 (m), 8.27 (td), 8.71 (dd), 8.96 (m)	<0.001	<0.001	0.914	0.908	0.002	<0.001
phenylalanine**	3.17 (dd), 3.30 (dd), 3.99 (dd), 7.32 (m), 7.33 (m), 7.38 (m)	0.043	<0.001	0.490	<0.001	0.551	<0.001
succinate**	2.40 (s)	0.593	1.000	<0.001	0.530	<0.001	<0.001
tyrosine**	3.06 (dd), 3.20 (dd), 3.95 (dd), 6.84 (d), 7.17 (d)	0.038	<0.001	0.257	<0.001	0.783	<0.001
valine**	0.99 (d), 1.05 (d), 2.28 (m), 3.62 (d)	0.226	<0.001	0.311	<0.001	0.997	<0.001

[a] * indicates $p < 0.05$ and ** indicates $p < 0.01$ as a result of one-way ANOVA. [b] Letters in parentheses denote the peak multiplicities: s, singlet; d, doublet; t, triplet; dd, doublet of doublet; td, triplet of doublet; q, quartet; and m, multiplet. [c] *p* values were results of Tukey post hoc pairwise multiple-comparison tests using SPSS 12.0K. [d] Abbreviations: AUS, Australia; KOR, Republic of Korea; NZ, New Zealand; USA, the United States.

OPLS-DA score plots derived from the ^{1}H NMR spectra of sirloin (or chuck) extracts shown in **Figure 4** provide a paired comparison between origin countries. OPLS-DA score plots (**Figure 4**) showed clear separation according to the first component (OPLS 1). The OPLS-DA models of beef samples between Australia and Korea (**Figure 4A**), Korea and New Zealand (**Figure 4D**), Korea and the United States (**Figure 4E**), and New Zealand and the United States (**Figure 4F**) were established using one predictive and one orthogonal component. The OPLS-DA models between Australia and New Zealand (**Figure 4B**) and Australia and the United States beef samples (**Figure 4C**) were established using one predictive and two orthogonal components and using one predictive and four orthogonal components, respectively.

To further understand the underlying variables contributing to the differentiation, we constructed the S-plot from the OPLS-DA

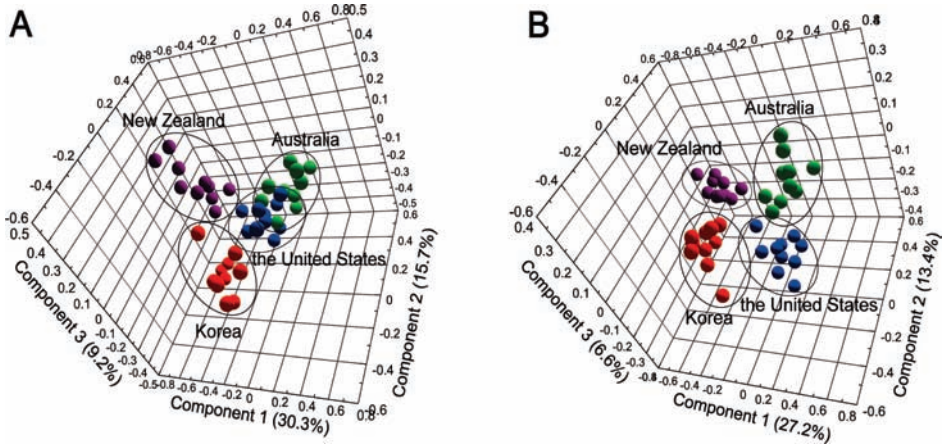

Figure 3. PCA (**A**) and OPLS-DA (**B**) 3D score plots derived from the ^{1}H NMR spectra of beef sirloin (or chuck) extracts obtained from Australia, Korea, New Zealand, and the United States.

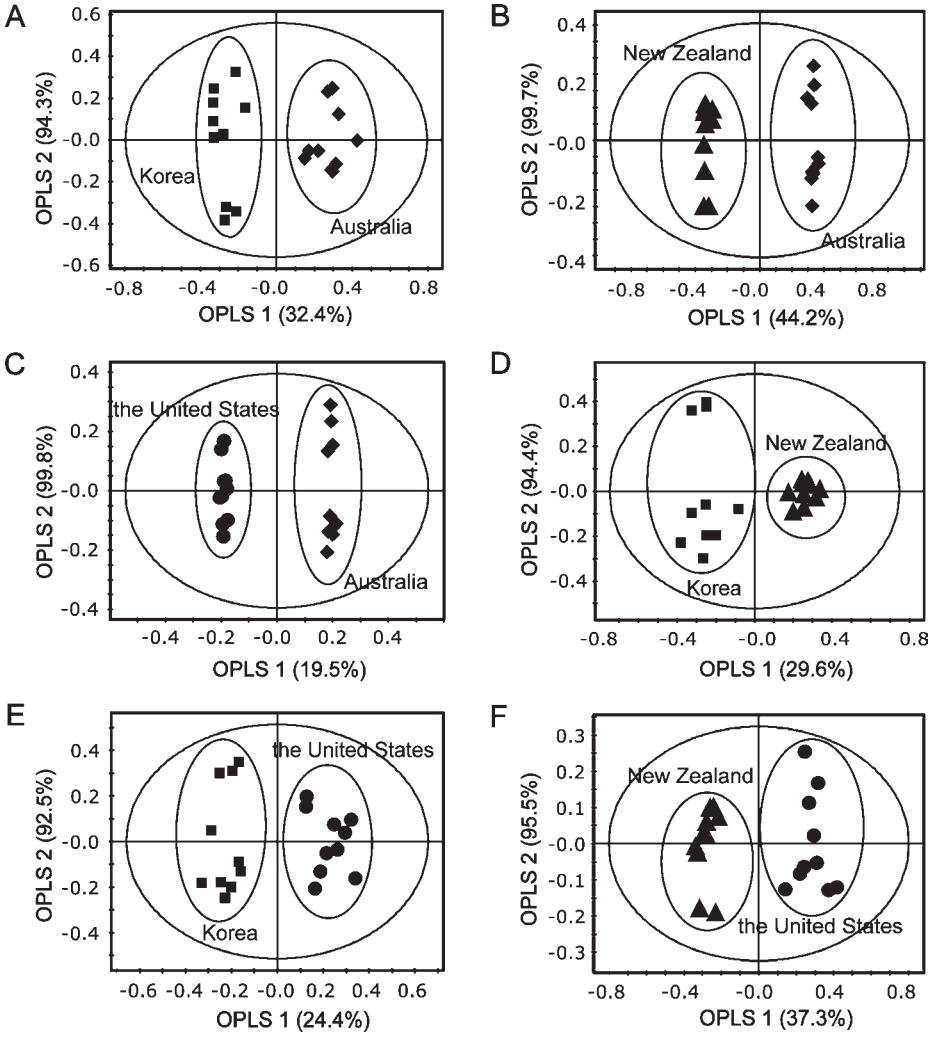

Figure 4. OPLS-DA score plots derived from the ^{1}H NMR spectra of beef sirloin (or chuck) extracts shown in pairs: (**A**) Australia and Korea (R_X^2 = 0.537, R_Y^2 = 0.943, and Q^2 = 0.886); (**B**) Australia and New Zealand (R_X^2 = 0.596, R_Y^2 = 0.997, and Q^2 = 0.967); (**C**) Australia and the United States (R_X^2 = 0.643, R_Y^2 = 0.998, and Q^2 = 0.871); (**D**) Korea and New Zealand (R_X^2 = 0.496, R_Y^2 = 0.944, and Q^2 = 0.884); (**E**) Korea and the United States (R_X^2 = 0.503, R_Y^2 = 0.925, and Q^2 = 0.840); (**F**) New Zealand and the United States (R_X^2 = 0.480, R_Y^2 = 0.955, and Q^2 = 0.867). The ellipse represents the 95% confidence region for Hotelling's T^2 (*50*).

model (**Figure 5**). The S-plot shows the covariance p against the correlation p(corr) variables of the discriminating component. The variables selected in the S-plot are highlighted with a dotted rectangle. Cutoff values for the covariance of $p \geq |0.05|$ and for the correlation of p(corr) $\geq |0.5|$ were used (*42*). Therefore, variables in the dotted rectangles of **Figure 5** contribute to the group separation and were considered as statistically significant metabolites. **Figure 5A** shows the most relevant variables affecting differentiation

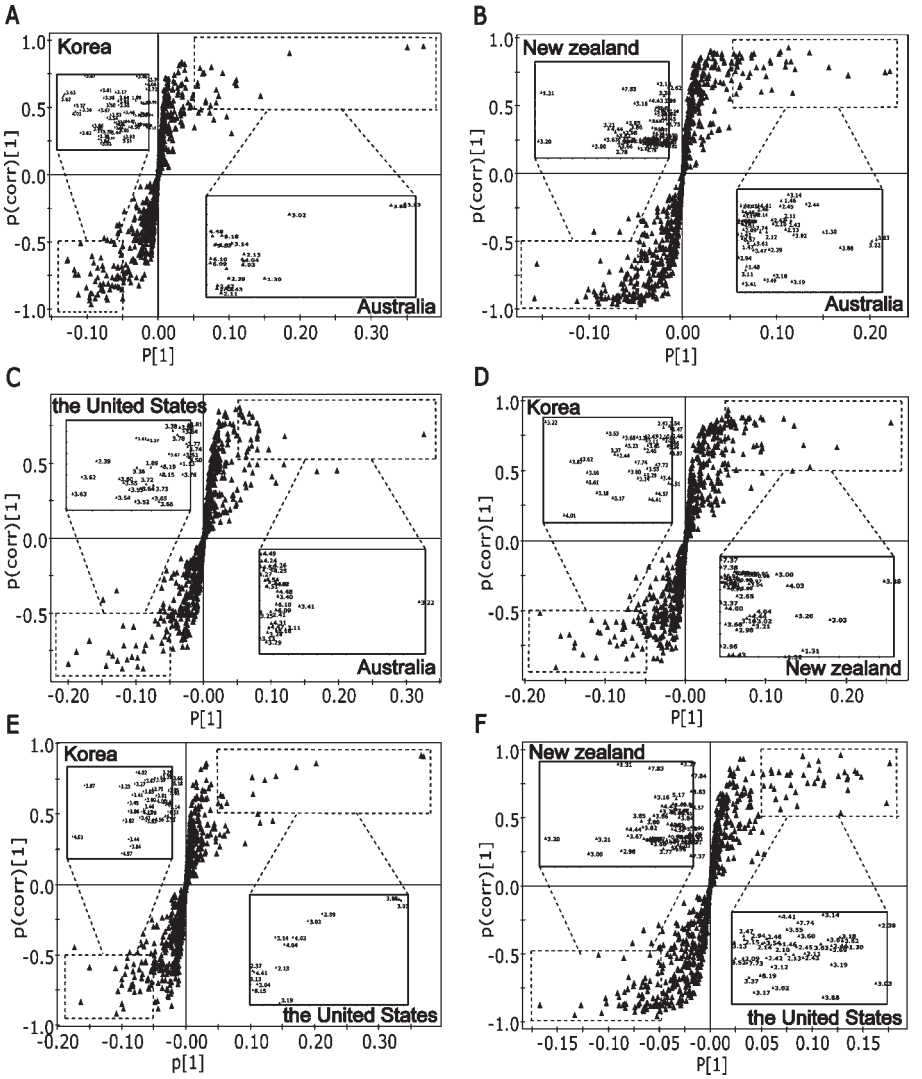

Figure 5. S-plot generated from OPLS-DA model: (**A**) Australia and Korea; (**B**) Australia and New Zealand; (C) Australia and the United States; (**D**) Korea and New Zealand; (**E**) Korea and the United States; (**F**) New Zealand and the United States. The range of the variables selected is highlighted with a dotted rectangle. Cutoff values for the covariance of $p \geq |0.05|$ and for the correlation of $p(\text{corr}) \geq |0.5|$ were used. The variables in dotted rectangles represent the metabolites responsible for differentiation in OPLS-DA score plots; the names of metabolites corresponding to the variable are given in the text.

between Australian and Korean beef. Australian beef samples were characterized by the higher levels of alanine (bin at 1.46 ppm), carnitine (bins at 2.42 and 2.43 ppm), creatine (bins at 3.02, 3.03, and 3.88 ppm), glutamine (bins at 2.11−2.13 ppm), and succinate (bin at 2.39 ppm) compared to Korean beef samples, whereas Korean beef samples were higher in acetate (bin at 1.89 ppm), betaine (bins at 3.85 and 3.86 ppm), creatinine (bins at 4.00 and 4.01 ppm), glycerol (bins at 3.52−3.57, 3.60−3.63, and 3.72−3.75 ppm), and glycine (bins at 3.50 and 3.51 ppm).

Alanine (bins at 1.46−1.48 ppm), carnitine (bins at 2.39−2.42, 3.22, 3.24, and 3.41 ppm), and glutamine (bins at 2.10−2.13 and 2.43−2.47 ppm) were more abundant in Australian beef samples; betaine (bins at 3.85−3.86 ppm), carnosine (bins at 2.60, 2.62, 2.65, 2.68, 2.98, 3.00, 4.43−4.46, and 7.83 ppm), creatine (bins at 3.89−3.90 ppm), glycerol (bins at 3.52−3.53, 3.74−3.75 ppm), glycine (bins at 3.50−3.51 ppm), isoleucine (bins at 0.93−0.95 and 1.02 ppm), leucine (bins at 0.96−0.99 ppm), phenylalanine (bin at 7.32 ppm), and valine (bins at 1.00−1.01 and 1.04−1.05 ppm) were more abundant in New Zealand beef samples (**Figure 5B**). Relative to the U.S. beef samples, alanine (bins at 1.46−1.47 ppm) and carnitine (bins at 3.22−3.24 and 3.40−3.41 ppm) were higher in Australian beef samples. In contrast, acetate (bin at 1.89 ppm), glycerol (bins at 3.52−3.55, 3.60−3.61, and 3.72−3.75 ppm), hypoxanthine (bins at 8.15 and 8.79 ppm), and succinate (bin at 2.39 ppm) were higher in the U.S. beef samples (**Figure 5C**). In comparisons between Korean and New Zealand beef samples, alanine (1.47−1.48 ppm), carnitine (3.22−3.24 and 3.41 ppm), glutamine (2.10−2.11 and 2.43−2.46 ppm), and glycerol (3.54−3.55 and 3.60−3.63 ppm) were relatively higher in the Korean beef samples. Conversely, carnosine (2.62, 2.65, 2.96, 2.98, 3.00, and 4.43−4.45 ppm), creatine (3.02−3.04 and 3.88−3.89 ppm), glutamate (2.36−2.37 ppm), glycine (3.50 ppm), isoleucine (0.93−0.95 and 1.02−1.03 ppm), lactate (1.31−1.32, 4.00, and 4.03 ppm), leucine (0.96−0.99 ppm), phenylalanine (7.32 and 7.37−7.38 ppm), and valine (1.00−1.01 and 1.04−1.05 ppm) were higher in New Zealand beef samples (**Figure 5D**). Betaine (3.27−3.28 and 3.85−3.87 ppm) and carnitine (3.23, 3.41, and 3.43 ppm) were more abundant in Korean beef samples, whereas creatine (3.02−3.03 and 3.88 ppm), hypoxanthine (8.15 ppm), and succinate (2.39 ppm) were more abundant in the U.S. beef samples (**Figure 5E**). Finally, carnosine (2.65, 2.67, 2.98, 3.00, 4.43−4.45, and 7.83−7.84 ppm), creatine (3.89−3.92 ppm), glycine (3.50 ppm), isoleucine (0.93−0.95 and 1.02−1.03 ppm), leucine (0.96−0.99 ppm), phenylalanine (7.32 and 7.37−7.38 ppm),

Table 3. Quantification of Identified Metabolites in Beef Sirloin (or Chuck) Extracts

	concentration mean ± standard error (μM)			
metabolite	Australia	Korea	New Zealand	United States
acetate	111.28 ± 14.21	261.32 ± 30.52	274.35 ± 13.29	252.43 ± 27.85
alanine	1237.91 ± 72.51	1163.71 ± 86.55	1087.79 ± 32.71	1147.17 ± 64.08
anserine	214.93 ± 49.55	556.86 ± 67.04	719.11 ± 50.49	411.01 ± 66.53
betaine	345.85 ± 38.4	375.31 ± 38.29	456.74 ± 30.58	315.07 ± 23.69
carnitine	1072.83 ± 69.97	1022.66 ± 63.87	983.18 ± 45.99	829.28 ± 38.28
carnosine	1802.96 ± 87.68	2492.74 ± 320.38	3447.54 ± 171.14	2187.41 ± 125.15
choline	76.73 ± 5.28	118.5 ± 13.32	125.57 ± 11.43	107.79 ± 4.65
creatine	8769.99 ± 413.59	7752.34 ± 490.5	10147.92 ± 171.42	8768.14 ± 196.21
creatinine	138.25 ± 10.52	242.65 ± 27.29	413.08 ± 15.73	189.32 ± 12.46
fumarate	123.02 ± 19.31	84.76 ± 11.89	160.89 ± 17.1	107.37 ± 14.42
glutamate	229.91 ± 25.7	241.62 ± 45.32	489.03 ± 20.47	393.05 ± 31.17
glutamine	1582.02 ± 237.99	1148.18 ± 201.58	476.93 ± 64.67	1508.29 ± 185.39
glycerol	903.03 ± 70.49	1515.35 ± 117.81	1401.6 ± 53.77	1732.09 ± 101.1
glycine	368.34 ± 26.45	379.36 ± 32.68	551.88 ± 18.61	402.07 ± 29.8
hypoxanthine	678.18 ± 58.48	1159.7 ± 135.04	1497.99 ± 57.51	1254.6 ± 88.57
inosine	301.14 ± 26.11	427.49 ± 33.47	395.56 ± 38.59	356.78 ± 41.47
isoleucine	46.75 ± 3.37	85.7 ± 14.97	215.92 ± 14.82	72.44 ± 10.27
lactate	14817.25 ± 555.7	17300.81 ± 1145.11	21406.66 ± 443.4	16764.97 ± 482.13
leucine	86.05 ± 6.13	150.85 ± 24.51	390.39 ± 33.32	138.21 ± 19.52
methionine	168.81 ± 20.31	148.53 ± 22.45	262.51 ± 12.18	151.61 ± 8.67
niacinamide	90.35 ± 6.44	135.28 ± 8.64	141.86 ± 6.17	96.76 ± 6.23
phenylalanine	54.02 ± 3.36	105.7 ± 18.05	242.58 ± 15.65	80.81 ± 10.96
succinate	167.81 ± 31.14	110.14 ± 35.65	172.53 ± 22.4	453.8 ± 37.98
tyrosine	48.4 ± 4.1	97.9 ± 13.13	251.11 ± 17.54	81.35 ± 11.06
valine	76.65 ± 5.44	115.1 ± 15.63	268.81 ± 19.22	111.25 ± 11.56

and valine (1.00−1.01 and 1.04−1.05 ppm) were higher in New Zealand beef samples, whereas alanine (1.46 ppm), glutamine (2.10−2.13, 2.43−2.44, and 2.47 ppm), glycerol (3.52−3.55 and 3.60−3.63 ppm), and succinate (2.39 ppm) were higher in the U.S. beef samples (**Figure 5F**).

To test the validity of the OPLS-DA model, we performed a permutation procedure using the PLS-DA model with the same number of components. Generally, the extrapolated intercept value of the $Q^2 > 0.05$ indicates overfitting in the original model. Therefore, these analyses show that our models are statistically valid (*43*, *45*, *46*). The permutation results are given in the Supporting Information, Figure 3S. Other external validation processes were also performed to validate our OPLS-DA models. For the prediction, we randomly left out three beef samples from each country group and built the OPLS-DA prediction model without them. These prediction results were able to correctly predict the origins of all test beef samples in three times processes (*45*, *46*) and are shown in the Supporting Information, Figure 4S.

Quantification of Metabolites. Metabolite concentrations were determined using the 600 MHz library from Chenomx NMR Suite 6.0, which compares the integral of a known reference signal (DSS) with signals derived from a library of compounds containing chemical shifts and peak multiplicities for all of the resonances of compound. Individual metabolite levels in the beef extracts are shown in **Tables 2** and **3**. Identified metabolite levels differed dramatically between beef samples from different countries. Acetate, anserine, choline, creatinine, glycerol, hypoxanthine, niacinamide, phenylalanine, and tyrosine levels differed significantly between Australian beef and Korean beef. Most of the detectable metabolite levels showed significant differences between Australian and New Zealand samples. Australian and U.S. samples showed differences in acetate, carnitine, glutamate, glycerol, hypoxanthine, and succinate levels. Korean and New Zealand beef samples exhibited different levels of carnosine, creatine, creatinine, fumarate, glutamate, glycine, isoleucine, lactate, leucine, methionine, phenylalanine, tyrosine, and valine. However, Korean and U.S. beef samples showed similar metabolomic profiles, differing only in their levels of glutamate, niacinamide, and succinate. Anserine, betaine, carnosine, creatine, creatinine, glutamine, glycine, isoleucine, lactate, leucine, methionine, niacinamide, phenylalanine, succinate, tyrosine, and valine levels were significantly different between New Zealand and U.S. beef. Notably, with the exception of alanine, carnitine, glutamine, glycerol, and succinate, most of the identified metabolites were substantially higher in New Zealand beef samples, especially the amino acids. In contrast, many of the metabolite levels were relatively low in Australian beef samples. U.S. samples exhibited the highest levels of succinate.

Considerable variability was detected among beef samples from different countries, suggesting that the metabolite levels and their relative composition were affected significantly by breed, feeding regimen, production system, pre- and postslaughter conditions, and environmental parameters, although some of the these factors cannot be fully elucidated. Possible sources of such variations can be found in the literature. Levels of both essential (i.e., valine, isoleucine, leucine, methionine, and phenylalanine) and nonessential (i.e., glutamine, glycine, tyrosine, glutamate, and alanine) amino acids differed significantly as a function of cattle breed (*48*). In addition, levels of free amino acids were increased during post-mortem aging, indicating some degradation of proteins and/or peptides (*34*), and there was a significant effect of diet on most of the amino acids. Silage-fed animals had higher concentrations of various amino acids compared to concentrate-fed animals (*31*). Also, many environmental factors such as diet, season, ambient temperature, and production system can influence fatty acid composition (*28*, *32*). Unfortunately, fatty acid composition was not observed in our ^{1}H NMR data of aqueous beef extracts. The biological functions of carnosine and anserine, dipeptides found in skeletal muscle and nerve tissue, are still obscure, and their levels in beef muscle differ significantly (*49*). Currently, the reasons for the differences in metabolomic profiles as a function of geographical origin are not fully understood. These differences, however, would clearly affect beef quality and food safety. Therefore, reliable analytical techniques are required to confirm the origins of beef samples.

Metabolite fingerprinting and profiling based on ^{1}H NMR spectra were used to analyze the similarities and differences among raw beef samples obtained from four countries with the aim of identifying markers useful for pinpointing geographical origin. This strategy has provided comprehensive information on a wide range of compounds including carbohydrates, amino acids, organic acids, and amines. The results shown here demonstrate that a combination of ^{1}H NMR and multivariate analyses allows comparisons of overall metabolite fingerprints and that this technique can be applied to conclusively identify differences between beef samples. Distinct separations between beef samples hailing from four different countries were observed in chemometric analyses using PCA and OPLS-DA, and several metabolites were identified as candidate biomarkers that could be used to quickly and easily differentiate beef samples. In this study, the relative content of succinate and various amino acids such as isoleucine, leucine, methionine, tyrosine, and valine could potentially serve as markers to distinguish between various raw beef samples. However, succinate, proposed as a biomarker candidate, is largely used as a substitute for salt in foods. Therefore, external factors such as feed type should be carefully considered for biomarker assessment.

In summary, the current study demonstrates that ^{1}H NMR-based metabolomic fingerprinting is a useful tool for distinguishing origins of raw beef samples and that its combination with chemometric analysis largely improves sample classification. A complementary approach using both nontargeted and targeted metabolite profiling was employed for discriminating the geographical origins of beef and identifying their potential biomarkers. Shintu et al. (*8*) has reported potential molecular markers of geographic origin of dried beef samples using HR-MAS NMR spectroscopy. On the other hand, we suggested metabolite profiling approaches in aqueous extracts of raw beef samples using solution NMR spectroscopy coupled with multivariate statistical analysis. Our studies demonstrate that the geographical origin of raw beef can be rapidly discriminated and major metabolites related to origin quantitatively evaluated using metabolite profiling. Reliable discrimination of biomarkers related to the geographical origin of raw beef is essential to both the consumer and producer. Further investigation of these metabolic fingerprints could lead to the establishment of discrimination biomarkers for geographic origin in the beef industry.

Supporting Information Available: Figures 1S−4S. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review June 8, 2010. Revised manuscript received August 22, 2010. Accepted August 28, 2010. This work was supported by grants from the Korean Ministry of Education, Science and Technology (2009-008146 and NRF-20090094024) and from the Korea Basic Science Institute (T3073).