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Rapid Determination of the Geographical Origin of Honey Based on Protein Fingerprinting and Barcoding Using MALDI TOF MS

JUN WANG,[†] MICHAEL M. KLIKS,[§] WEIYUE QU,[†] SOOJIN JUN,[‡] GONGYI SHI,[‖] AND QING X. LI*,[†]

[†]Department of Molecular Biosciences and Bioengineering and [‡]Department of Human Nutrition, Food and Animal Sciences, University of Hawaii of Manoa, Honolulu, Hawaii 96822, [§]CTS Foundation, 3081G Paty Drive, Honolulu, Hawaii 96822, and [‖]Bruker Daltonics, Inc., 2859 Bayview Drive, Fremont, California 94538

The authentication of foods is an important aspect of quality control and food safety. Honey is one of the most natural and most popular foods in the world. A fast and reliable method to determine the geographical origin of honey was developed based on fingerprinting and barcoding of proteins in honey by using matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI TOF MS) and MALDI Biotyper 1.1 software, respectively. The protein mass spectra of 16 honey samples of known Hawaii origin were obtained and peak information was extracted to generate protein fingerprints. This information was transformed into a database library of spectral barcodes that were used for differentiation of the geographical origin of honeys based on pattern matching. The differentiation ability of the database library of barcodes was validated by comparing the results of replicate assays of 5 of the 16 honey samples of known Hawaii origin obtained directly from the producers. Validation results showed that the protein fingerprints of honeys have better comparability with those honeys in the library known to be from the same region than with those of honey samples from other regions. The protein fingerprints were used to differentiate the geographical origins of commercially purchased honey samples with labels indicating that they were produced in different countries and various states of the USA, including Hawaii. The results showed that the MALDI TOF MS Biotyper system can be a rapid, simple and practical method for determining the geographical origin of honeys sold in commerce.

KEYWORDS: Honey; protein; barcode; Biotyper; MALDI TOF; geographical origin; food quality

INTRODUCTION

Honey is a nutritious and healthy natural food produced by honey bees from plant nectars. Honey consists mostly of the sugars glucose and fructose, as well as maltose, sucrose, water and other minor components including proteins, organic acids, amino acids, vitamins, flavonoids, and acetylcholine (*1*). Food authentication is an international issue in quality control and food safety. Regulatory authorities, food processors, retailers, and consumers are interested in knowing the origin and quality of foods. The deliberate mislabeling and adulteration of foods, particularly honeys, are matters of increasing global concern. The constituents of honey naturally vary under different climatic and environmental conditions (*1*−*4*). Therefore, the detection of natural or deliberate adulteration is of considerable interest to both consumers and regulatory authorities.

*Corresponding author: Qing X. Li, 1955 East-West Road, Honolulu, HI 96822. Tel: 808-956-2011. Fax: 808- 956-3542. E-mail: qingl@hawaii.edu.

Several compounds naturally found in honeys have been used to determine their geographical and botanical origin. For example, different volatile and semivolatile organic compounds have been analyzed to detect variations related to the floral origins and the processing of honey (*5*−*7*). Saccharides represent the main components of honeys, and relatively more information is available in the literature on them as indicators of the economic adulteration of honeys (*8*−*10*). Enzymatic activities have been used to indicate the floral origins of honeys (*11*). Analyses of fermentation products such as glycerol and ethanol also have been used to derive information about the processing of honey. However, these analytes did not distinguish floral or geographical sources of honey (*7*, *12*, *13*). Analyses of minerals and trace elements in honeys could be suitable for the determination of geographical origin, due to the fact that the presence of these analytes is usually associated with environmental pollution (*7*, *14*, *15*). Honeys from different floral sources or geographical regions may contain different organic acids that could be used to provide information on the geographical and floral origins of honey samples (*16*). Profiles of phenolic acids, phenolic esters and

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Table 1. Origin, Floral Source, and Protein Content of 16 Honey Samples of Known-Hawaii Origin[a]

no.	sample sites	floral and nectar source	protein content (mg/g)
1	Puako, Hawaii County	kiawe (*Prosopsis* sp.)	0.82
2	South Kona, Hawaii County	Christmas berry (*Schinus* sp.)	0.93
3	South Kona, Hawaii County	mixed floral	0.70
4	North Kona, Hawaii County	'Ohi'a lehua (*Metrosideros* sp.)	0.69
5	Puna/Hamakua, Hawaii County	'Ohi'a lehua (*Metrosideros* sp.)	0.83
6	Kane'ohe, Honolulu County	mixed floral	0.67
7	Kailua, Honolulu County	mixed floral	0.91
8	Nanakuli valley, Honolulu County	Kiawe (*Prosopsis* sp)	0.79
9	Waikane valley, Honolulu County	mixed floral	0.68
10	Farm-10, Honolulu County	mixed floral	0.71
11	Farm-11[b], Kaua'i County	mixed floral	0.86
12	Farm-12[b], Kaua'i County	mixed floral	0.74
13	Farm-13[b], Kaua'i County	mixed floral	0.92
14	Farm-14[b], Kaua'i County	mixed floral	0.77
15	Farm-15[b], Kaua'i County	mixed floral	0.86
16	Farm-16[b], Kaua'i County	mixed floral	0.85

[a] Mixed floral nectar sources may include *Hibiscus, Ficus, Passifloria, Waedelia, Prosopsis, Schinus, Brasaia, Metrosideros* sp., macademia and various eucalyptus species. [b] These samples were purchased directly from apiculture farms in Kaua'i County, and specific farm names are not identified for proprietary reasons.

aromatic carbonyl compounds in honeys could be indicative of the botanical origin of honey (*7*). The different amino acids in honey have been used to differentiate their geographical sources (*17*, *18*).

The use of each of these strategies has limitations for accurately differentiating the geographical origin of a given honey sample. For example, volatile trace compounds are difficult to isolate from honeys and are not suitable for determining their geographical origins. Saccharides in honeys can be indicators of commercial adulteration, but not for the differentiation of their floral or geographical origins. Enzyme activities and fermentation products can give some information about the processing and storage conditions of honeys, but not about their origins. Analyses of minerals and trace elements found as contaminants in honey can broadly suggest a region of origin. Likewise profiles of different organic acids and amino acids commonly occurring in honeys can give some indirect information as to their origins.

A variety of proteins are present in trace amounts in honeys, and their presence has been shown to be a useful indicator of the geographical and floral origins of honeys (*7*, *14*, *15*, *19*). Because different regions have distinct and characteristic floral communities, using proteins to differentiate the floral and geographical origins of honey may be more productive than using other compounds. In recent years, some work has been done on the geographical or floral origin of honey based on trace proteins (*19*−*23*). Marshall and Williams (*23*) demonstrated at least 19 distinct protein bands in honeys of different floral origins using silver-staining sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE).

Matrix assisted laser desorption ionization tandem time-of-flight mass spectrometry (MALDI TOF MS) integrated with MALDI Biotyper 1.1 software (Bruker Dalatonics, Billerica, MA) has been used as a rapid, simple, and reliable method for the classification and identification of microorganisms (*24*). This suggested its potential for applications in clinical and environmental diagnostics and food quality control. Subsequent studies have shown that this technique has the advantages of high speed, good sensitivity, high accuracy and precision, and simple operation (*25*, *26*) although the instrument cost is quite high. To our knowledge, studies using MALDI TOF MS for determination of the geographical origin of different honey samples have not been reported. The objective of the present study was to investigate the use the protein pattern matching capabilities of the MALDI TOF MS Biotyper 1.1 system as a tool for elucidating the geographical origins of honeys.

MATERIALS AND METHODS

Honey Samples. Sixteen honey samples were acquired directly from apiculture operations in Hawaii and were considered to be of known Hawaii origin. Glucose and 38 additional commercial honey samples with labels indicating various regional origins, including 15 labeled as being of Hawaii origin, were purchased from local retail markets (**Tables 1** and **2**). Glucose was dissolved in water at 75% to make a synthetic glucose plasma sample for protein recovery studies. All samples were stored at 4 °C until analyzed.

Protein Extraction. Proteins were extracted according to the method of Won et al. (*19*). In brief, 20 g of honey was suspended in 20 mL of distilled water. Proteins were extracted by overnight shaking at 4 °C. After centrifugation at 10,000 rpm for 60 min at 4 °C, the supernatant was filtered through a 0.45 μm filter. The pellet and filtrate were collected and combined in a dialysis bag (molecular weight cutoff > 1000 Da) and then dialyzed against 1 L of double-distilled water with 2 changes per day for 2 days to remove low molecular weight impurities such as sugars. Concentrations of proteins were determined according to the Bradford method (*27*, *28*).

SDS−PAGE. SDS−PAGE was performed as described by Lee et al. (*29*). In brief, protein samples (each 30 μg) were mixed with the sample buffer and heated at 100 °C for 5 min. The denatured proteins were separated on 4−20% gradient SDS−PAGE mini-gels (9×10 cm^2, PAGEr Gold Precast Gel, Cambrex Bioscience, Rockland, ME) following by Coomassie dye (G250) staining for 1 h. To determine molecular weight, 10 μL of precision plus protein standard (BioRad, CA) were applied on the gels. After electrophoresis, gels were destained overnight in a solution containing 25% (v/v) methanol and 10% (v/v) acetic acid.

MALDI TOF MS. The MALDI sample matrix was α-cyano-4-hydroxycinnamic acid (HCCA) dissolved in 30% aqueous acetonitrile containing 0.1% trifluoracetic acid (TFA) at a concentration of 5 g/L. An aliquot of 1 μL of protein HCCA solution was manually spotted on a prepared MTP standard target plate (Bruker Daltonics). The droplet was air-dried at room temperature, and the sample was analyzed on a Bruker Daltonics UltraflexIII TOF/TOF MS equipped with a Smartbeam laser (1−200 Hz). Mass spectra of singly and multiply charged ions were obtained in linear positive ion mode with the source 1 and the source 2 voltages set at 25 kV and 23.6 kV, respectively. A high voltage pulse generator supplied a voltage pulse to the MALDI sample plate at a predetermined time after the laser pulse. The time elapsed between the voltage pulse and the time that ions are detected was recorded by the digitizer to produce a time-of-flight spectrum. Based on a standard mixture of 1,000−10,000 Da proteins for calibration, the pulse voltage was optimized at 1.5 kV in this study. The detector voltages were set at

Table 2. Summary Information of Sites and Protein Content of 38 Commercial Honey Samples Labeled as Being from Different Regions of the World

sample	origin of sample	protein content (mg/g)	sample	origin of sample	protein content (mg/g)
		Different Countries			
H1	Supha Bee Farm, Thailand	0.34	H8	Japan	0.49
H2	Supha Bee Farm, Thailand	0.42	H9	Milburn, New Zealand	0.78
H3	Supha Bee Farm, Thailand	0.37	H10	New Zealand	0.66
H4	Ontario, Canada	0.51	H11	Vietnam	0.23
H5	Jakarta, Indonesia	ND[a]	H12	North Eastern China	0.13
H6	Jakarta, Indonesia	0.18	H13	Bhutan	0.33
H7	Kent, U.K.	0.62	H14	Bhutan	0.27
		Different States of the U.S.A.			
H15	Virginia	0.63	H27	Hawaii	0.64
H16	California	0.49	H28	Hawaii	0.52
H17	Wisconsin	0.67	H29	Hawaii	0.41
H18	North Dakota	0.42	H30	Hawaii	0.76
H19	Maryland	0.78	H31	Hawaii	0.68
H20	Alaska	0.52	H32	Hawaii	0.31
H21	Alaska	0.45	H33	Hawaii	0.66
H22	California	0.53	H34	Hawaii	0.73
H23	Washington	0.64	H35	Hawaii	0.59
H24	Hawaii	0.62	H36	Hawaii	0.44
H25	Hawaii	0.58	H37	Hawaii	0.67
H26	Hawaii	0.73	H38	Hawaii	0.76

[a] Not detected.

1.4 kV. Ions in a range of 1,000 to 10,000 m/z were detected at high resolution with a suppression mass gate set at 500 m/z, to prevent detector saturation from matrix cluster peaks, and an extraction delay of 500 ns. Spectra were automatically acquired with Bruker Flexcontrol software with fuzzy control of laser intensity. The same algorithm allowed the automatic optimization of laser energy taking into account both the lower and higher intensity threshold limits (set up to 0% and 100%, respectively) during detection. In addition, the instrument was externally calibrated with a mixture of protein standards with a detection limit of 50 μg/mL of each. To evaluate the precision and reproducibility of MALDI TOF measurements, each protein extract was run 4 times and all mass spectra were processed and analyzed identically with MALDI Biotyper 1.1 software.

Quality Control and Quality Assurance. All sample pretreatment and analytical procedures were performed using quality assurance and quality control measures. Laboratory blanks (i.e., spot plate, solvent, and matrix) were analyzed in the same way as the samples. Analytical blanks consisted of 3 matrix, solvent and spot plate blanks. If no significant difference was found (t test, $p \leq 0.05$) between analyte concentrations in the analytical blanks, background contamination was considered to be negligible during storage and analysis. Limits of detection (LODs) for analytes in samples were set as signals 3 times greater than the standard deviations of the average background signals of the blanks.

Evaluation of protein recoveries was done using fortified glucose plasma samples. An amount of 10 mg of bovine serum albumin (BSA) was added to 20 g of each of the 5 synthetic glucose plasma samples that had almost the same density as honey. The fortified samples were extracted and analyzed in the same manner as the honey samples.

Data Analysis. Mass spectra were converted into bidimensional images ("biology mass barcodes") by analyzing the resulting peak lists with MALDI Biotyper 1.1 software. Protein mass spectra profiles were obtained from 16 honey samples known to be of Hawaii origin. MALDI Biotyper 1.1 software was used to extract a list of peaks from each honey sample's mass spectrum profile and transform the peaks into patterns resulting in a database library of honey protein barcodes. Protein peaks extracted from the 38 commercial honey samples were transformed into barcodes that were then compared with the barcodes in the mass spectral database library obtained from the 16 samples of known Hawaii origin. Match scores were obtained by comparing the masses and signal intensities of proteins detected in test samples with those in the mass spectra database library. These scores were used to rank the confidence of the assay results obtained from commercial honey samples. To improve the confidence of the mass spectra database library, the MALDI Biotyper 1.1 software corrects the mass deviation of peaks using a sophisticated recalibration algorithm (*24*). The peak picking function offers an initially accepted error window and a desired adjustment result. This functionality makes the MALDI Biotyper identification exceptionally robust and accurate (*24*). Principal component analysis (PCA) was done to distinguish differences among commercial honey samples in 2- and 3-dimensional scatter plots.

RESULTS AND DISCUSSION

Extraction of Proteins from Honey. Reproducible, quantitative extraction of all proteins from honey is the first, important step in the process used in this study. Average recoveries of BSA from synthetic glucose plasma samples were between 70 ± 10% and 110 ± 10%. The results suggest that the protein extraction method used is appropriate.

The protein content in the 16 honey samples of known Hawaii origin was very similar, ranging from 0.67 to 0.93 (mean: 0.80) mg/g (**Table 1**). The protein concentrations in the 38 commercial honey samples labeled as originating in different geographical regions, including Hawaii, North America, and Asia, varied widely from nondetected to 0.78 (mean: 0.52) mg/g (**Table 2**). Protein concentrations were found to be very low, from nondetected to 0.42 mg/g (mean: 0.26), in the 9 commercial honey samples that were labeled as having originated in Bhutan, China, Indonesia, Thailand, and Vietnam (**Table 2**). Protein concentrations in the honeys labeled as originating in Japan and Canada were also relatively low, at 0.49 and 0.51 mg/g, respectively. Further research is needed to determine if some of these honeys had been ultrafiltered, ultracentrifuged, diluted with other sugars, or otherwise processed or adulterated.

SDS−PAGE of the 16 honey samples acquired directly from apiculture operations in Hawaii and the 15 samples purchased in retail markets labeled as being of Hawaii origin showed major bands of protein with masses of approximately 29 kDa and 45 kDa as well as minor bands of lower molecular weight proteins (**Figure 1**). MALDI TOF MS protein ion mass spectra were transformed into protein ion mass spectral barcodes as illustrated in **Figure 2** that shows the mass spectrum (peaks in the 1,000 to

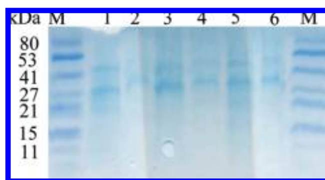

Figure 1. SDS−PAGE of proteins extracted from honey. Lane M is the protein markers. Lanes 1, 3, and 4 are honeys of known Hawaii origin, and lanes 2, 5, and 6 are commercially acquired honeys labeled as being of Hawaii origin.

10,000 *m*/*z* region) and resulting barcode from known Hawaii honey sample number 9. The MALDI TOF MS protein mass spectrum (**Figure 2A**) was first adjusted and normalized into peak lists (**Figure 2B**) by MALDI Biotyper 1.1 software. The peak mass deviations were corrected through a sophisticated recalibration algorithm and the adjusted, normalized peak lists were then transformed into barcodes (**Figure 2C**). Comparison of bands on SDS−PAGE with MALDI TOF mass spectra suggests that most proteins produce multiply charged polypeptide ions during the process of MALDI TOF analysis.

Effect of Sample Preparation on the Production of Multiply Charged MALDI Ions. Multiply charged MALDI ions can be generated by varying the experimental conditions such as the type of matrix and matrix solution used, the matrix/analyte ratios, crystallization conditions, and the sample deposition method (*30*, *31*). Liu and Schey (*30*) reported that using matrices with higher ionization energy, such as HCCA, resulted in a much higher yield of multiply charged protein ions. When the matrix and analyte were cocrystallized on polyether ether ketone, an "electron free" surface, a larger proportion of multiply charged ions was produced, compared with the usual stainless steel surface (*30*). Cohen and Chait (*32*) found that formation of high molecular weight analyte ions was favored in a matrix solution composed of 2:3:1 (v:v:v) of formic acid:water:acetonitrile (or isopropanol or methanol), particularly when a slow matrix crystal growth method was utilized. Zhou and Lee (*33*) observed that highly charged protein ions were produced by formation of a protein-doped HCCA matrix in the presence of glycerol on the top of a previously deposited pad. Kononikhin et al. (*34*) found that the charge state and signal intensity were related to the method of sample spotting. For instance, using electrospray deposition of the analyte resulted in multiply charged ions being produced at threshold laser irradiance. Demirev et al. (*35*) showed that use of multiply charged precursor ions in MALDI TOF/TOF experiments for protein identification enriched the yield of protein ions and simultaneously enhanced database searching results.

Sample preparation is critical for producing singly and multiply charged MALDI ion fragments during MALDI TOF MS analysis. The common spot-sample methods include dried-droplet, thin-layer, and sandwich preparation. Among them, thin-layer preparation has much higher sensitivity in the detection of proteins (*30*). In the present study, the thin-layer sample preparation method was adapted based on a slight modification of the work of Liu and Schey (*30*) that has maximized the yield of multiply charged protein ions. This method includes (1) applying 1 μL of matrix solution (HCCA saturated in ethanol) on a stainless steel target to create a thin layer of matrix; (2) mixing the sample with HCCA saturated in 0.1% TFA and 30% acetonitrile (1:1, v:v); and followed by (3) applying 1 μL to form a thin layer that is air-dried at room temperature. In addition, most of the mass profiling studies performed with MALDI TOF MS have been restricted to peptides and small proteins (<20 kDa) because the sensitivity of the standard ion detectors

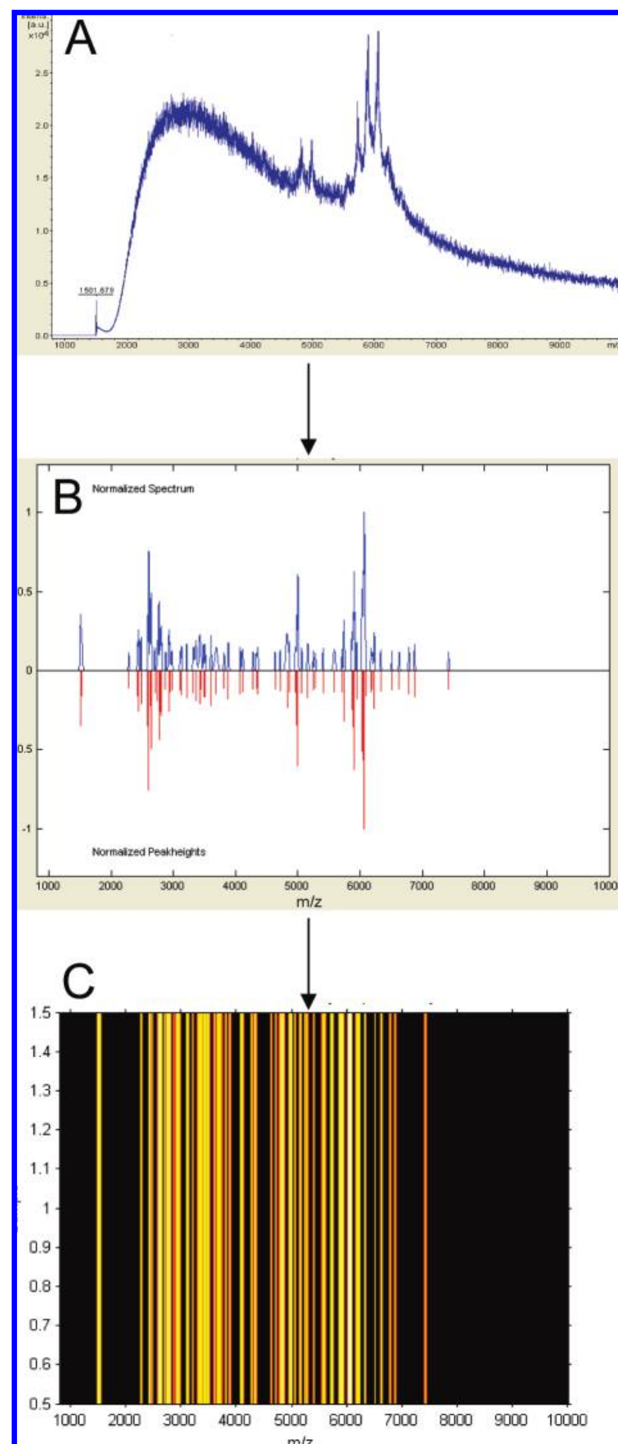

Figure 2. Transformation process of protein ion mass spectral barcodes from a MALDI TOF MS spectrum of proteins extracted from honey sample 9 from Waikane valley, Honolulu County. (**A**) MALDI TOF MS raw mass spectrum. (**B**) Graphic output of the identification results displayed within the graphic view. The upper part shows the unknown spectrum containing matching peaks within the defined mass tolerance windows and non-matching peaks. The lower part shows the dedicated main spectrum. (**C**) The peaks transformed into barcodes.

decreases with increasing ion mass (*36*). Therefore, in this study, MALDI TOF MS were restricted to peptides and small proteins (<20 kDa).

Proteins are often enzymatically digested prior to MALDI TOF MS determination. In the present study enzymatic digestion of extracted proteins was eliminated to simplify the procedure

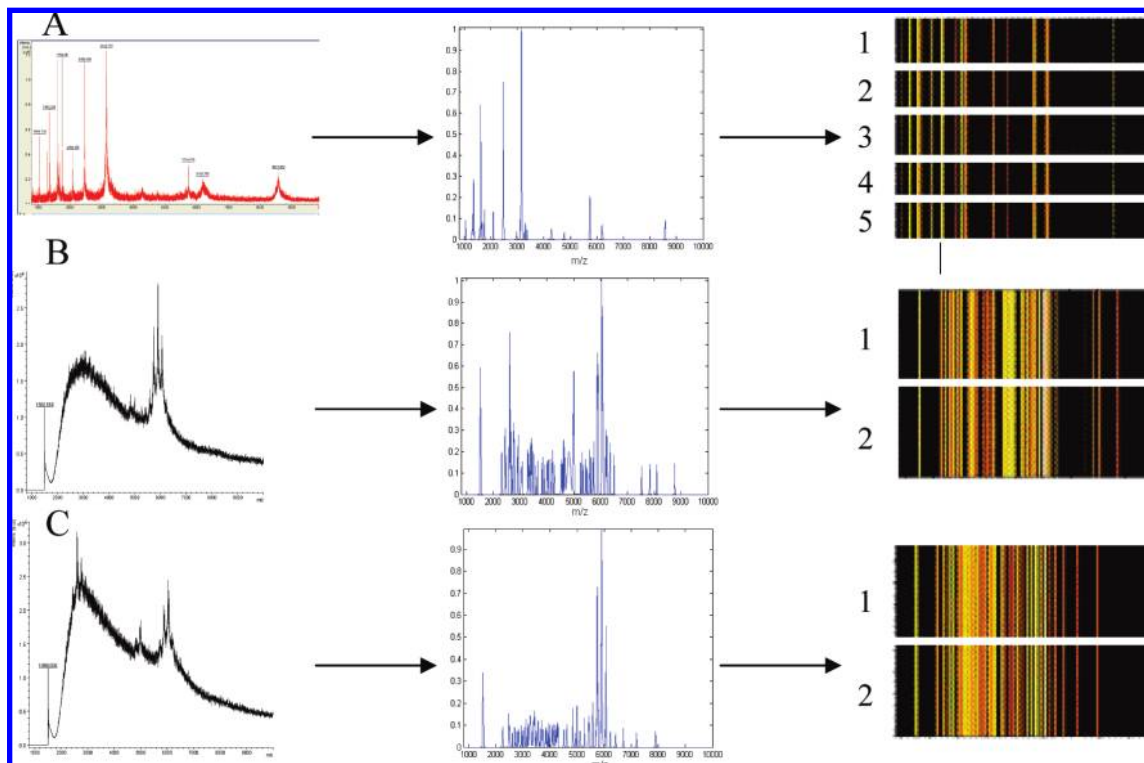

Figure 3. Peak picking results using MALDI Biotyper software. (**A**) One of the five MS spectra (five laser shot events on the same sample spot) of a mixture of protein standards, its corresponding adjusted normalized mass spectrum, and the five barcode spectra. (**B**) One of the two MS spectra (two sample spots of the same sample) of honey sample 4 from North Kona, Hawaii County, its corresponding adjusted normalized mass spectrum, and the two barcode spectra. (**C**) One of the two MS spectra (two sample spots of the same sample) of honey sample 7 from Kailua, Honolulu County, its corresponding adjusted normalized mass spectrum, and the two barcode spectra.

and reduce processing time. We used protein mass spectra fingerprinting of singly and multiply charged MALDI mass fragments in the 1,000 to 10,000 m/z range from extracted intact proteins, followed by barcoding, to discriminate patterns among honey samples of different geographical origins.

Method Reproducibility and Precision. To evaluate the reproducibility and precision of this method, the identical standard protein mixture was analyzed 5 times and all mass spectra were processed identically and analyzed with MALDI Biotyper 1.1 software. Peak picking generated a normalized and baseline corrected peak list vector for each spectrum. Peak picking results showed no obvious differences among resulting barcodes representing the 5 replicate analyses of the mixture of protein standards (**Figure 3A**).

Reproducibility experiments were performed under the same conditions using the same mixed protein standards. The spectra obtained from 3 assays repeated over 1 week were virtually identical (data not shown). When duplicate spots of each of the protein extracts from 2 honey samples of known Hawaii origin (numbers 4 and 7) were analyzed in the same manner, peak picking results between the 2 replicate spots were apparently identical (**Figures 3B** and **3C**), indicating good reproducibility and precision of the automatic peak picking function of MALDI Biotyper 1.1 software. In subsequent experiments, MALDI TOF MS coupled with the MALDI Biotyper 1.1 software was used to elucidate differences in honey samples labeled as being of various geographical origins. As with the protein standards, mass spectra fingerprinting of the singly and multiply charged MALDI protein mass fragments from these samples were detected in the 1,000−10,000 m/z range and visualized as barcodes.

Development of a Honey Protein Mass Spectra Database Library of Barcodes. The honey samples acquired directly from 16 apiculture operations in Hawaii were used to produce a database library of protein mass spectra barcodes. **Figure 4** shows MALDI TOF protein mass spectra transformed into barcodes representing protein profiles from these 16 honeys known to be of Hawaii origin. Similarities and differences among the protein mass spectra barcodes are evident. A few common peaks produced by ions in the 4,500 to 6,500 m/z range occurred in all mass spectra. Proteins producing ions in the 8,500 to 10,000 m/z range were present in some honeys, but almost absent in the others. The results demonstrate that protein content differs among honey samples of different floral and geographic origins and that these can be readily visualized using MALDI TOF MS and Biotyper 1.1 software. Samples tested included single nectar sourced honeys from the islands of O'ahu such as kiawe (*Prosopsis*; samples 1 and 8), and Christmas berry (*Schinus;* sample 2), and Hawaii, such as 'ohi'a lehua (*Metrosideros;* samples 4 and 5). Honeys from mixed nectar sources such as *Hibiscus*, *Ficus*, *Passifloria*, *Waedelia*, *Prosopsis*, *Scheffleria*, *Eucalyptus*, *Macadamia*, *Psidium*, *Mangifera*, *Persea*, *Litchi*, and *Schinus* were obtained from apiculture farms on O'ahu and Kaua'i.

The reproducibility of the results of the MALDI TOF MS method for determining the geographic origin of honey was evaluated by repeat MALDI TOF MS analyses of the 5 known Hawaii origin honey samples. The resulting barcodes of repeat analyses (**Figure 4B**) apparently are identical to the corresponding barcodes produced when these honeys were analyzed the first time (**Figure 4A**, also enlarged in **Figure 4C**). These results indicate that honey protein mass spectra profiles can be transformed into unique barcodes using MALDI TOF MS which confirms the presence of both common protein ions, diagnostic for honey in general, and of unique patterns of ions that characterize honeys of different geographic origins.

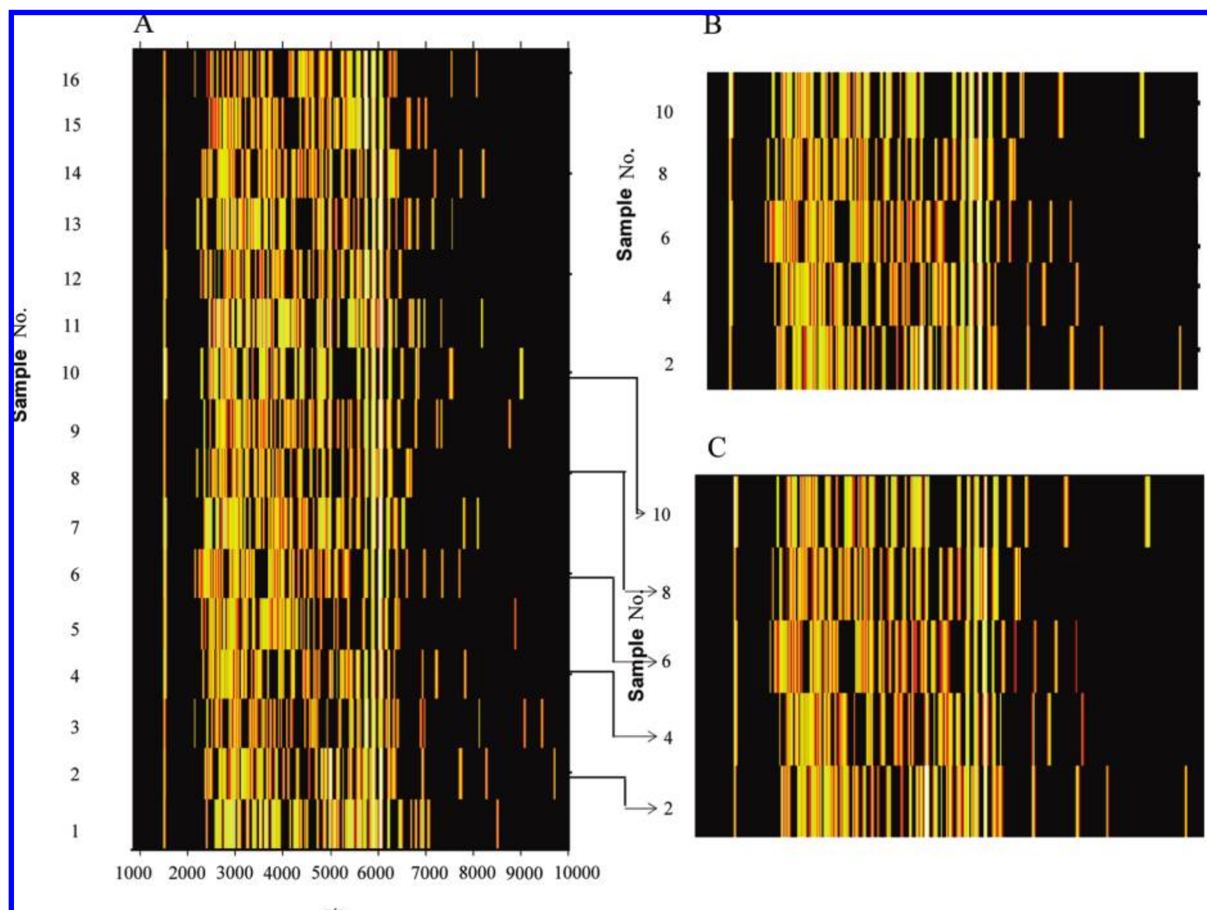

Figure 4. (**A**) MALDI TOF protein mass spectra barcodes of the 16 honeys of known Hawaii origin. (**B**) The protein barcodes of repeat analyses of the Hawaii origin honey samples 2, 4, 6, 8, and 10. (**C**) Enlarged display of the protein barcodes of the samples 2, 4, 6, 8, and 10 in the database library.

Differentiation of Commercial Honeys Labeled As Being of Hawaii Origin. To effectively differentiate honeys actually produced in Hawaii from others it is essential to obtain the protein mass spectra of honeys known to have been produced in Hawaii, convert them into mass spectra barcodes and compare those with the barcodes in the database library. In the present study MALDI TOF mass spectra of protein extracts were obtained by quadruple measurements of the 16 original honeys under the same conditions. The 16 honey protein mass spectra were averaged automatically to generate peak lists from the entire spectral standards. Seventy main peaks in a spectral mass range of 1,000 to 10,000 m/z were processed by using automatic peak picking function of the MALDI Biotyper 1.1 software.

The precision of the protein mass spectra database library was tested by comparison among the peak lists of the 16 honeys of known Hawaii origin. Correlation analysis of the resulting peak lists was ranked using the Biotyper identification operation. When the protein mass spectra of the 16 honeys of known Hawaii origin honeys in the database library were averaged for the first 70 main peaks, correlation coefficients (R^2) between each of the 16 database library spectra and the average spectrum ranged from 0.77 to 0.95. Such information indicates the usefulness of the 70 main peaks (m/z) for differentiation of Hawaii-origin honeys from others.

The R^2 values of protein ion mass spectra peaks from 38 commercially purchased honeys labeled as being from a variety of production regions, including 15 from Hawaii, 9 from 7 other states of the U.S.A., and 14 from 9 other countries are displayed in **Table 3**. Comparability analysis, between these honeys and the database library of protein mass spectra created using the 16 honeys of known Hawaii origin, showed R^2 values between 0.80 and 0.93 for 13 of the 15 commercial honey samples with a label

Table 3. Correction Coefficients (R^2) between 38 Commercial Honey Samples (Including 15 Commercially Labeled Hawaii-Origin Honey Samples) and the Mass Spectral Database of the 16 Known Hawaii Origin Honeys

no.	origin	R^2	no.	origin	R^2
		Different Countries			
H1	Supha Bee Farm, Thailand	0.28	H8	Japan	0.42
H2	Supha Bee Farm, Thailand	0.39	H9	Milburn, New Zealand	0.82
H3	Supha Bee Farm, Thailand	0.14	H10	New Zealand	0.79
H4	Ontario, Canada	0.79	H11	Vietnam	0.23
H5	Jakarta, Indonesia	0.70	H12	North Eastern China	0.50
H6	Jakarta, Indonesia	0.33	H13	Bhutan	0.43
H7	Kent, U.K.	0	H14	Bhutan	0.21
		Different States of the U.S.A.			
H15	Virginia	0.49	H27	Hawaii	0.77
H16	California	0.62	H28	Hawaii	0.92
H17	Wisconsin	0.64	H29	Hawaii	0.90
H18	North Dakota	0.66	H30	Hawaii	0.84
H19	Maryland	0.81	H31	Hawaii	0.89
H20	Alaska	0.27	H32	Hawaii	0.75
H21	Alaska	0.38	H33	Hawaii	0.92
H22	California	0.61	H34	Hawaii	0.93
H23	Washington	0.47	H35	Hawaii	0.84
H24	Hawaii	0.81	H36	Hawaii	0.87
H25	Hawaii	0.80	H37	Hawaii	0.92
H26	Hawaii	0.91	H38	Hawaii	0.86

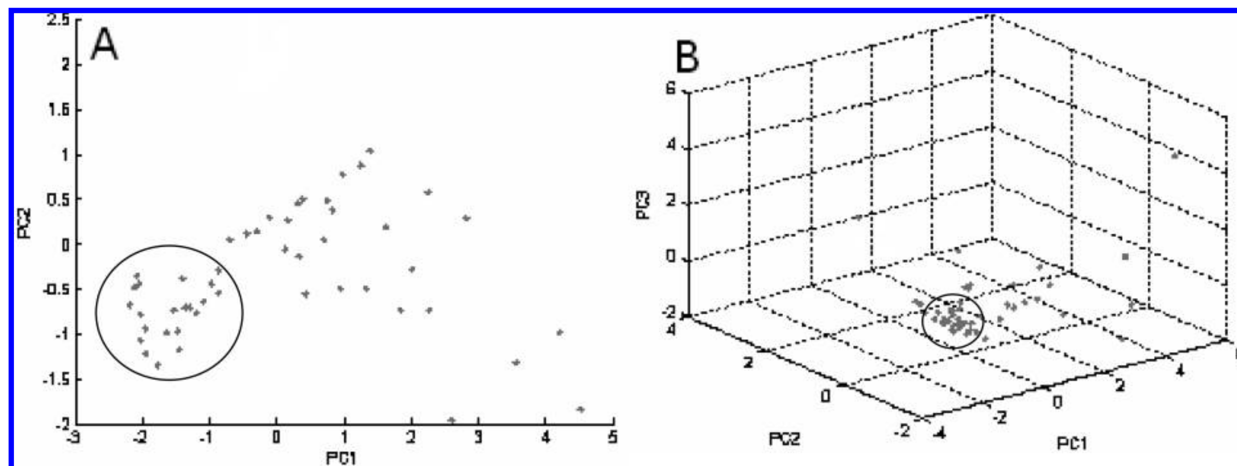

Figure 5. Two-dimensional (**A**) and three-dimensional (**B**) displays of the principal component analysis (PCA) of protein mass spectra profiles derived from 38 commercial honeys. Fifteen of the 38 were labeled Hawaii origin (inside of the circle) and those labeled as being from 7 other states and 9 other countries (outside of the circle).

indicating Hawaii origin. The R^2 values of 2 samples were somewhat lower (H27, R^2 0.77 and H32, R^2 0.75). Low R^2 values suggest that the honey may not be produced in Hawaii or it may be adulterated even though it has a label indicating Hawaii origin.

These results suggest that MALDI TOF MS coupled with the Biotyper 1.1 software can be a rapid, simple, and cost-effective method for differentiating honey sources from relatively small production regions. These techniques can be adapted to detect deliberate processing such as by heating, ultrafiltration, ultracentrifugation, dilution and economic adulteration with other sugars.

The results show very low correlation between the honeys of known Hawaii origin and honeys produced in different states in the United States or other countries. The R^2 values of honeys from 7 states ranged from 0.27 to 0.81 and from 0 to 0.82 in honeys from 9 other countries (**Table 3**). Given a sufficiently representative protein mass spectral database library drawn from honeys from target regions, protein mass spectra profiles, particularly when expressed as easily read barcodes, can be used to rapidly assess honeys in commerce as to the veracity of their labeling.

High R^2 values of protein mass spectra peaks occurred between the honey sample from Canada (R^2 0.79) and the 2 New Zealand honeys (R^2 0.79 and 0.82) and the mass spectra peaks from the 16 of known Hawaii origin honeys used to generate the Hawaii honey database library. This coincidence may reflect similarities among the subtropical and temperate floral sources foraged by bees within the greater Pacific region. The purposeful blending of honeys from several geographical sources, such as commonly occurs in commerce, will alter the protein mass spectra profiles in the resulting honey products.

PCA of protein fingerprints, expressed as barcodes, clearly segregated honeys of different geographical and floral origins. Commercially purchased honeys labeled as being of Hawaii origin (15 samples) were clustered together (**Figure 5**). Most honey samples labeled as being from different geographic regions were well separated in "regional" clusters. These methods should have a wide variety of other uses such as the detection, classification and identification of microorganisms, in clinical, forensic, and environmental diagnostics, and in food safety research, product regulation, and as a tool for quality control and quality assurance testing in the food-processing industry (*24*).

In summary, the present study demonstrated a new methodology for using protein mass spectra profiles to authenticate the purity and geographical origin of honey in commercial trade. MALDI TOF MS protein profiles from some Hawaii origin honeys have been used as a database to discriminate honey origins. To our knowledge, using protein barcodes for differentiation of honey origins has not been reported. The method includes 3 simple steps: rapid protein extraction, MALDI TOF MS analysis, and transformation of protein mass spectra into barcodes with the MALDI Biotyper 1.1 software. The results showed that the MALDI TOF MS Biotyper system is an excellent alternative method for creating protein profiles of honeys in commerce in order to identify geographic origin and they suggest that these techniques can be applied to determining the country of origin of other foods.

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