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Quantification of coffee blends for authentication of Asian palm civet coffee (Kopi Luwak) via metabolomics: A proof of concept

Udi Jumhawan,[1,3] Sastia Prama Putri,[1] Yusianto,[2] Takeshi Bamba,[1,3] and Eiichiro Fukusaki[1,*]

Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan,[1] Indonesian Coffee and Cocoa Research Institute, Jalan PB Sudirman 90, Jember East Java 68118, Indonesia,[2] and Division of Metabolomics, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan[3]

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Asian palm civet coffee (Kopi Luwak), an animal-digested coffee with an exotic feature, carries a notorious reputation of being the rarest and most expensive coffee beverage in the world. Considering that illegal mixture of cheap coffee into civet coffee is a growing concern among consumers, we evaluated the use of metabolomics approach and orthogonal projection to latent structures (OPLS) prediction technique to quantify the degree of coffee adulteration. Two prediction sets, consisting of certified and commercial coffee, were made from a blend of civet and regular coffee with eleven mixing percentages. The prediction model exhibited accurate estimation of coffee blend percentage thus, successfully validating the prediction and quantification of the mixing composition of known–unknown samples. This work highlighted proof of concept of metabolomics application to predict degree of coffee adulteration by determining the civet coffee fraction in blends.

[**Key words:** Asian palm civet coffee; Kopi Luwak; Metabolomics; Orthogonal projection to latent structures; Coffee blend; Prediction model]

Coffee is one of the most popular beverages in the world. Among the diverse varieties of commercially marketed coffee, Asian palm civet coffee or Kopi Luwak (Indonesian words for coffee and palm civet) has developed a reputation as one of the world's priciest and rarest coffee (1–3). This animal-treated coffee is made from coffee berries that have been eaten by the Asian palm civet (*Paradoxurus hermaphroditus*), which uses its keen senses to select the best and ripest berries. The transient fermentation inside the civet's gut hypothetically adds a distinct flavor to the coffee beans. As a result, its rarity as well as the coffee's exotic and unique production process ultimately accounts for its high selling price, which is around US $200/lb, approximately a hundred times higher than regular coffee (International Coffee Organization, http://www.ico.org/prices/pr-prices.pdf).

An important concern related to the price gap between civet and regular coffees is the growing attempt of fraud involving illegal mixture of cheaper coffee into premium civet coffee. It is therefore essential to develop robust methods to determine the ratio of civet coffee in blends. Quality evaluation of coffee has been conventionally assessed on the basis of human sensory perception and visual examination (4–7). However, this method tended to be highly subjective with only up to 20% precision (7), unavoidably expensive and only feasible for raw bean. Therefore, an alternative, instrument- and non-human-based measurement method to estimate the quality of coffee similar to the previously developed protocols for various food and agricultural products should be established (8–11).

An effective and reliable method to differentiate civet coffee from regular coffee has been reported previously based on the identification and quantification of biochemical markers through metabolomics (1,3). Metabolomics analysis to detect and quantify coffee adulteration, i.e., Arabica–Robusta fractions in coffee blends, have been stated employing a wide range of analytical instruments (7,12–14). The main purpose of this study is to further evaluate the applicability of biochemical markers of civet coffee to predict the degree of coffee adulteration on the basis of the mixing ratio by means of orthogonal projection to latent structures (OPLS) analysis. A set of coffee blends with known-ratio was used to construct a prediction model and subsequently evaluated to predict the known–unknown samples. The model was proven to be robust for accurate estimation ratio of known-unknown coffees and provides proof of concept for the approach developed in this report.

MATERIALS AND METHODS

Coffee beans and chemicals Two sets of coffee blends from authentic (certified) and commercial coffee were prepared to construct the prediction model. Authentic samples of civet coffee and regular coffee (not having gone the digestive tract of civet) were acquired from the Indonesian Coffee and Cocoa Research Institute (ICCRI). Civet coffee and regular coffee from Sidikalang, Indonesia, which have been well known for its scarcity and unique aroma (15), were acquired commercially. The samples were ground and mixed in different mixing proportions to obtain representative blends. Ultimately, samples with 1 g of pure civet coffee, pure regular coffee, and blends of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, and 90% civet coffee were made. These groups of samples were referred to as training or experimental set.

* Corresponding author. Tel./fax: +81 6 6879 7424.
E-mail address: fukusaki@bio.eng.osaka-u.ac.jp (E. Fukusaki).

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For validation, two sets of coffee blends were made from same authentic and commercial civet coffees with two unknown regular coffees (acquired commercially), with 25% and 75% mixing ratio. The validation set was analyzed simultaneously in a separate week to investigate the influence of expected variance resulting from day-to-day measurements. This sample set was then projected to the model as test set to verify its performance for prediction of mixing ratio of known–unknown samples. The list of samples is described in Table S1.

High-grade solvents were used for the sample preparation. The chemical standards for identification and their vendor were as follows: pyroglutamic acid (ICN Biomedicals, OH, USA), sucrose (Kisida chemical, Osaka, Japan), caffeic acid (TCI chemical, Tokyo, Japan) and caffeine, glycolic acid, chlorogenic acid, quinic acid (Sigma–Aldrich, Milwaukee, WI, USA). To quantify the concentration of significant metabolites, chemical standards, vendors, and purities were as follows: citric acid (Nacalai-Tesque, Kyoto, Japan, 99.5%) and malic acid (Nacalai-Tesque, 99%).

Sample preparation and GC/MS analysis After adding ribitol internal standard (60 μL, 0.2 mg/mL with deionized water), the pure and blended samples (15 mg) were subjected to metabolite extraction followed with chemical derivatization utilizing methoxyamine hydrochloride and MSTFA based on previous report (3). All samples were analyzed in four replicates ($n = 4$). Analysis of coffee bean extract was performed on a GCMS-QP 2010 Ultra (Shimadzu, Kyoto, Japan) fitted with a 30 m × 0.25 mm i.d. fused silica capillary column coated with 0.25 μm low bleed, CP SIL 8 CB column (Varian Inc., Palo Alto, CA, USA). GC and MS conditions were identical to our prior study (3). The coffee samples and blank (only extraction solvent) were analyzed in a randomized order. Concentration of selected significant metabolites in coffee blends was calculated using the linear calibration curve between concentration of chemical standards (adjusted to 1, 10, 50, 100, 250, 500, 750 and 1000 μM with the extraction solvent) and peak area. Details of quantitative analysis were explained elsewhere (3).

Data handling, preprocessing and statistical analysis Data handling including peak detection and multiple alignments of the retention times were carried out utilizing software package, MetAlign (http://www.wageningenur.nl/nl/Expertises-Dienstverlening/Onderzoeksinstituten/rikilt/show/MetAlign.htm). Metabolite identification was completed in similar fashion with previous report (3) utilizing information of mass spectral fragmentation pattern, retention indexes and retention times that correspond with in-house mass spectral library and further confirmed with NIST library. Conventionally, training and test set was integrated into total data set prior to preprocessing [normalization to peak intensity of ribitol, internal standard, and scale to unit variance (UV)].

To minimize effect of unwanted factors interfering analysis of training and test set, subset-wise scaling was also applied in addition to the conventional data handling. In subset-wise scaling, each data set, training and testing set, was thought as one separate unit, preprocessed independently and incorporated into total data set (Fig. S1). Normalization and scaling were done in similar manner with conventional method. Lastly, the data matrix was then subjected to SIMCA-P version 13 (Umetrics, Umea, Sweden) for multivariate analysis.

RESULTS AND DISCUSSION

Blended samples were divided into training and test sets to develop a robust prediction model. Prediction model was constructed from training set of 11 blends from coffees with known identities. Test set comprised of blend of known and unknown coffees to validate the prediction model for estimation of mixing ratio.

Fig. 1 illustrates the total ion chromatogram of the pure and blended samples of coffee bean extract. Both pure and blended

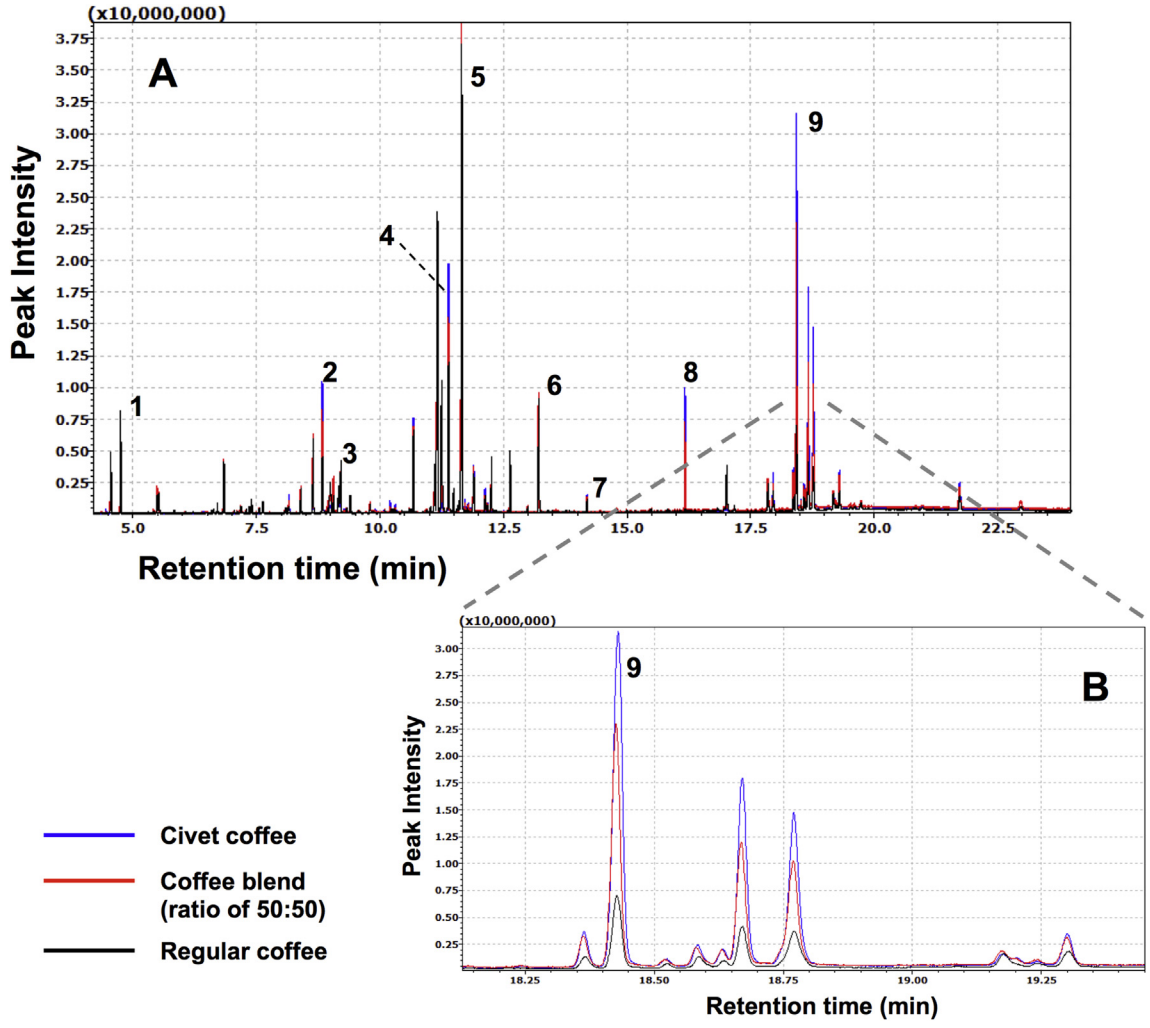

FIG. 1. Total ion chromatograms of the pure and coffee blend samples with 50:50 ratio (A). Inset shows substantial differences in the peak intensity among pure and coffee blends (B). Representative peak annotations: 1, glycolic acid; 2, malic acid; 3, pyroglutamic acid; 4, citric acid; 5, quinic acid; 6, inositol; 7, caffeic acid; 8, sucrose; 9, chlorogenic acid.