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Article

# Metabolic Profiling, Antioxidant Activity, and Alpha-Glucosidase Inhibitory Activity of the Roasted Beans of Luwak (Civet) Coffee

Lizma Febrina, Nizar Happyana,* and Yana Maolana Syah

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**ABSTRACT:** Metabolite profiling of the roasted coffee beans fermented by wild and caged civets (luwak) was assessed by $^{1}$H NMR-based metabolomics. Our result indicated that the metabolite profile of the roasted civet coffee beans possessed uniqueness and was distinct from the regular coffee. According to OPLSDA data, the roasted civet coffee beans contained higher levels of acetic acid, lipid, trigonelline, quinic acid, citric acid, and malic acid than those of regular coffee. The most discriminant metabolites in the caged civet coffee were lipid and acetic acid. Meanwhile, malic acid and citric acid were the distinguishing metabolites in the roasted beans of wild civet coffee. Furthermore, the antioxidant properties and alpha-glucosidase inhibitory activity of the coffee samples were evaluated. The biological activities of the civet coffee beans were better than that of regular coffee. According to the PLS analysis, malic acid was the highest positive correlated with the antioxidant activity and alpha-glucosidase inhibition of the coffee sample. The possibility of coffee fermentation in the civet digestive tract increases malic acid levels so that it has a beneficial effect on its alpha-glucosidase inhibitory activity.

**KEYWORDS:** *roasted civet (luwak) coffee, metabolomics, $^{1}$H NMR, antioxidant activity, alpha-glucosidase activity, multivariate analysis, malic acid, OPLSDA*



## INTRODUCTION

Luwak (civet) coffee is one of the most expensive and rarest coffee in the world.[1] This coffee originates from coffee cherries fermented in the digestive system of the Asian palm civet (luwak, *Paradoxurus hermaphroditus*), which utilizes its keen senses to identify the best and ripest coffee cherries.[2] The fermentation process results in civet coffee having a unique taste different from that of regular coffee. It suggests that the metabolite profile of the coffee beans changes after fermentation in the civet digestive system.

Identification of civet coffee metabolites has been reported previously, with either a proximate analysis or a metabolomic approach. The proximate analysis showed that civet coffee contains less protein and caffeine but more fat when compared to regular coffee.[3] However, this method focused on only specific metabolites. Meanwhile, metabolomics provides high-throughput and concurrent systematic profiling of various metabolites in an organism.[4] Commonly, metabolomics is performed with both qualitative and quantitative analyses (metabolic profiling) but occasionally is carried out with only the qualitative technique (metabolic fingerprinting).[5] In previous studies, metabolomic fingerprinting of civet coffee had been presented using UV−vis and fluorescence spectroscopy.[6−8] This method successfully classified the metabolite content of civet coffee from its regular coffee based on the emission−excitation and UV absorption patterns. Conversely, this method could not describe the metabolite profile of civet coffee. GCMS-based metabolomics was applied to identify the profile of the volatile and acidic compounds in civet coffee, including caffeic acid, glycolic acid, quinic acid, pyroglutamic acid, inositol, chlorogenic acid, and sucrose.[9,10] However, this method could not be applied directly to analyzing the nonvolatile compounds. Derivatization is needed to enhance the volatility of the metabolites.

The other analytical technique employed for the metabolic profiling of civet coffee is NMR spectroscopy. This method has successfully identified and quantified several metabolites in green civet coffee beans without separation, such as alanine, asparagine, GABA, malic acid, chlorogenic acid, quinic acid, choline, sucrose, caffeine, and trigonelline.[11] This instrument has also been used to identify civet coffee metabolites dissolved in methanol.[12] The findings revealed that civet coffee had higher levels of fatty acids than regular coffee. Easy sample preparation is one of the fundamental advantages of NMR spectroscopy. This method enables the analysis of the sample without sample separation or preparation. It is profitable for compounds that are difficult to be ionized or require derivatization for GC−MS. Besides this, NMR is non-destructive, so the sample can be reused for reanalysis using another instrument or for another purpose.[13] Even so, the investigation of metabolic profiling of roasted civet coffee beans, especially metabolites dissolved in water using the NMR approach, has never been reported previously.

Roasting leads to chemical reactions, such as pyrolysis, caramelization, and Maillard reactions.[14] Consequently, the

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roasting process alters the metabolites of green coffee beans. Identification of the metabolites of roasted coffee beans using the metabolomic approach had been performed using a variety of instruments, including IR,[15] GC−MS,[16] LC−MS,[17] and $^1$H NMR.[18] According to the metabolomic results, each roasted coffee has a distinct profile of metabolites. For instance, Preanger-Java coffee has higher trigonelline, arabinose, and chlorogenic acid levels than Gayo coffee. Meanwhile, Gayo coffee has higher levels of acetic acid, lactic acid, and lipids than Toraja-Sulawesi coffee and Bajawa-Flores coffee.[18] The uniqueness of the roasted coffee metabolite profile affects the taste of each coffee, including the unique taste of civet coffee. To the best of our knowledge, information about the metabolite profile of roasted beans of civet coffee, both wild- and caged civet coffee, is still limited.

The antioxidant activity of coffee has been reported using in vitro tests, including the DPPH, ABTS, FRAP, and ORAC methods.[19] In addition, the ability to scavenge free radicals in cells and mice induced by alkyl peroxyl radicals was also previously reported.[20] This antioxidant activity provides benefits for preventing degenerative diseases, including diabetes mellitus.[21] Furthermore, coffee is also described as having the ability to inhibit the enzyme alpha-glucosidase.[22] Inhibiting alpha-glucosidase delays the breakdown of sucrose, preventing the development of postprandial diabetes and thus lowering the diabetic risk.[23]

The inhibition of the alpha-glucosidase activity and antioxidant properties of coffee is inextricably linked to the contained metabolites. In roasted coffee, the composition of polyphenols due to the Maillard reaction affects the antioxidant activity.[24] Meanwhile, chlorogenic acid,[25] trigonelline,[26] and malic acid[27] were reported to have both antioxidant properties and the alpha-glucosidase inhibitory activity. The concentration of the active compounds is likely to change after fermentation in the civet digestive system. Nevertheless, our literature study revealed that the antioxidant activity and alpha-glucosidase inhibitory activity of the roasted beans of luwak coffee are still limited.

Our previous study evaluated the metabolic profiles and in vitro antidiabetic properties of the green beans of luwak coffee.[11] As a continuation of our research, here, we applied $^1$H NMR-based metabolomics for analyzing the metabolic profile of the roasted civet coffee beans, including the caged and wild civet coffee and regular Arabica coffee for comparison. Proton signal assignment was performed using $^1$H NMR, further confirmed with 2D NMR, including COSY, TOCSY, and J-resolved spectroscopy. A quantitative $^1$H NMR analysis was performed to determine the concentrations of several identified metabolites. Orthogonal least-squares discriminant analysis (OPLSDA) was applied to classify the metabolome of the roasted civet coffee samples. *S*-plot was analyzed to reveal the specific metabolites for each coffee sample. The alpha-glucosidase inhibitory activity and antioxidant properties of the roasted coffee samples were evaluated as well. The correlation between the metabolite profile of the coffee samples and the bioactivities was investigated using partial least-squares (PLS).

## MATERIALS AND METHODS

**Materials.** Three types of roasted Arabica coffee beans of sigararutang variety, including wild civet coffee, caged civet coffee, and regular Arabica coffee, were analyzed in this report. These roasted coffee beans originated from the green coffee beans obtained from Rahayu Farmer Group, Pangalengan, Bandung, West Java. The coffee varieties on the plantation have been certified by the Directorate General of Plantations, Ministry of Agriculture, Republic of Indonesia, under permit no. 65/Kpts/Sr.120/2/2014. On this plantation, both wild and caged civets are treated humanely so that they can continue to produce the highest quality coffee. In this study, systematic random sampling was used from the coffee population of each group. The coffee population at issue is the coffee harvest on the plantation, which includes the harvest of wild civet coffee, caged civet coffee, and regular Arabica coffee. Figure S1 provides a summary of the sampling method. The green coffee beans were roasted for 8 min in a Behmor 1600 plus system (Behmor Inc., United States) with P2 mode to acquire a medium degree of roasting and then air-cooled for 1 h. The roasted coffee beans were ground with Encore (Baratza, Taiwan) and deposited in the Organic Chemistry Laboratory, Bandung Institute of Technology, in sealed tubes at −30 °C before the analysis. The chemicals used in this study, including 3-(trimethylsilyl)- 2,2,3,3-tetradeuteropropionic acid sodium salt, ferric chloride, deuterium oxide, methanol, sodium acetate trihydrate, ethanol, and acetic acid, were bought from Merck (Darmstadt, Germany). Acarbose was obtained from TCI (Tokyo, Japan). 1,1-Diphenyl-2-picryl hydrazyl (DPPH), ascorbic acid, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salts (ABTS), 2,4,6 tripyridyl-*s*-triazine (TPTZ), potassium persulfate, *p*-nitrophenyl-alpha-D-glucopyranoside (PNPG), and alpha-glucosidase from *Saccharomyces cerevisiae* were bought from Sigma-Aldrich (St. Louis, USA).

**Extraction for NMR Analysis.** $D_2O$ (1 mL) containing 1.0 mM TSP in phosphate buffer (pH 6.0) was added into a microtube (2 mL) containing 200 mg of the roasted coffee powder. The mixture was vortexed for 1 min and sonicated for 10 min. Afterward, it was incubated in a water bath at 95 °C for 30 min and then centrifuged for 6 min at 12.000 rpm in an MC-12 microcentrifuge (Benchmark, New Jersey, USA). Around 400 $\mu$L of the supernatant was moved into a new microtube and mixed with 100 $\mu$L of a $D_2O$-containing phosphate buffer. After that, the mixture was transferred to an NMR tube.

**NMR Parameter Analysis.** The NMR spectra of coffee samples were recorded using an INOVA-500 MHz NMR spectrometer (Agilent Technologies, Santa Clara, United States). The $^1$H NMR spectrum measurement was conducted using the presaturation mode with 128 scans, an acquisition time of 2.72 s, yielding 8012 Hz spectra width, a delay time of 2 s, and 64k data points. A shimming procedure was performed to ensure that the resulting peaks were satisfactory. ACD/Laboratories 12.0 software (Toronto, Canada) was used to process the NMR FID data further. To validate the detected signals in the $^1$H NMR spectrum, 2D NMR techniques, including $^1$H−$^1$H correlation spectroscopy (COSY), J-resolved spectroscopy, and total correlation spectroscopy (TOCSY), were applied. 2D NMR experiment was performed using 4 scans and 256 increments for COSY and TOCSY, while 128 increments for J-resolved measurement.

**Relative Quantification Analysis.** The metabolite concentration was computed by comparing the integral of the specified signal of the target metabolites to the area under the curve of the TSP signal (reference standard). In this study, the concentration of TSP was used as 1 mM. TSP is utilized as a reference standard because the signal is a singlet and lacks overlap, making it amenable to quantification.

Metabolite concentrations are shown as the mean value and the standard deviation for each of the six replicates. One-way analysis of variance (ANOVA) and Duncan's multiple range tests were used in Microsoft Excel to figure out how different the groups were (Microsoft Office 365). A significance level of 0.05 was judged to be statistically significant.

**Multivariate Statistical Analysis.** All roasted coffee $^1$H NMR spectra were centered and bucketed with a width of 0.04 ppm across the entire range of 0.50−10.00 ppm using ACD/Lab software. The water signal area located at 4.75−5.29 ppm was removed from the analysis. The obtained data set was normalized to the overall area of the bucket to eliminate measurement-induced bias. SIMCA software version 12.0 (Umetrics, Umea, Sweden) was utilized for multivariate data analysis by applying Pareto scaling and the OPLSDA technique. The data from the NMR spectrum are represented by the variable *X*


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(predictor) in the OPSL-DA model, whereas the data from the test group are represented by the *Y* (response) variable. Model accuracy was measured via the R2Y value, while prediction accuracy was measured by Q2 in a 200 permutation test used to validate the final model.

**Preparation of Samples for the Antioxidant and Alpha-Glucosidase Inhibitory Testing.** Solid−liquid extraction with deionized water (Barnstead Easypure II UF Deionization System) extracted all roasted coffee samples; 40 mL of deionized water was used to extract 5 g of the roasted bean mill. The proportion of powder/water is 1:8. For 20 min, the samples were sonicated. They were incubated at 90 °C for 30 min, allowed to cool in water for 10 min, and separated by centrifugation at 12.000 rpm for 6 min. A freeze-dryer (Bench Top Freeze-Dryer FDB-5503, Operon, Korea) was utilized to dry the obtained supernatant.

**In Vitro Antioxidant Test.** *Scavenging Activity of DPPH• Radicals.* The antioxidant activity of the roasted coffee sample was assessed using the DPPH• radical (DPPH•) scavenging assay. We combined 2 mL of each coffee sample with 2 mL of a methanol-based 0.004% DPPH• solution. It was then homogenized, allowed to keep warm for 30 min at 27 °C, and then the absorbance was recorded at the wavelength of 522 nm using a UV−vis spectrophotometer (Halo DB-20 s UV−vis double beam spectrophotometer, Dynamica, United Kingdom). The measurements were carried out in triplicate for each coffee sample, and ascorbic acid was used as the positive control throughout the experiment. The following equation determined the DPPH• scavenging effect of the sample under consideration

$$\%\ \text{Scavenging activity of DPPH radical} = \left(\frac{\text{abs}_{\text{control}} - \text{abs}_{\text{sample}}}{\text{abs}_{\text{control}}}\right) \times 100\%$$

$\text{Abs}_{\text{control}}$ represented the absorbance of the DPPH• solution, and methanol was used as a blank. Meanwhile, $\text{Abs}_{\text{sample}}$ represented the absorbance of the roasted coffee sample in the DPPH• solution. The linear regression linking the roasted coffee concentration and the percentage of the DPPH• scavenging effect was applied to ascertain the $IC_{50}$ value. In order to calculate the $IC_{50}$ value, the sample concentration was varied between 15 and 50 mg/L.

*Scavenging Effect of ABTS•+ Radical Cation.* The ABTS•+ radical cation was synthesized by adding potassium persulfate (140 mM) to a 7 mM ABTS•+ solution. After 16 h of incubation at room temperature in darkness, 250 μL of the mixed solution was diluted with 22 mL of ethanol. After thoroughly combining the roasted coffee sample with the ABTS•+ solution for 3 min, the absorbance was measured using a UV−vis spectrophotometer (Halo DB-20s UV−vis double beam spectrophotometer, Dynamica, United Kingdom). The measurements were taken at a wavelength of 734 nm. The following equation was used to determine the ABTS•+ scavenging effect

$$\%\ \text{scavenging activity of ABTS radical cation} = \left(\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}\right) \times 100\%$$

$\text{Abs}_{\text{sample}}$ denotes the absorbance of the roasted coffee samples, and $\text{Abs}_{\text{control}}$ denotes the absorbance of the control, which was an ABTS•+ solution and ethanol mixture. The positive control used was ascorbic acid. Each coffee sample and the positive control were measured in triplicate. The sample concentration was varied between 15 and 50 mg/L so that the $IC_{50}$ value could be calculated accurately. The percentage of the ABTS•+ radical cation scavenging effect and concentration of the sample were analyzed using linear regression to determine the $IC_{50}$ value.

*FRAP Test.* The TPTZ (2,4,6-tripyridyltriazine) solution, 2.5 mL of $FeCl_3 \cdot 6H_2O$ solution, and 10 mL of 300 mM sodium acetate buffer (pH 3.6) were combined to create the FRAP reagent, which was then warmed (preheated) at 37 °C prior to application. The FRAP reagent was combined with the roasted coffee sample and then incubated for 10 min at 37 °C in the dark. The absorbance was measured using a UV−vis spectrophotometer (a Halo DB-20 s UV−vis double beam spectrophotometer from Dynamica in the United Kingdom) at 593 nm. The equation was used to determine antioxidant activity according to previous methods.[11] Ascorbic acid was utilized as a positive control. The $EC_{50}$ value was computed using linear regression concerning the percentage of the antioxidant capacity and the concentration of the roasted coffee. The sample concentration was adjusted to between 15 and 50 mg/L to determine the $EC_{50}$ value. The concentration of a sample exhibiting 50% FRAP capacity is recognized as the $EC_{50}$ value of FRAP capacity.

*Inhibition of Alpha-Glucosidase Tests.* The roasted coffee sample was dissolved in a solution of methanol and water in a ratio of 1:9, respectively. Furthermore, 3.5 μL of the roasted coffee sample was enhanced to 10 μL of glucosidase (1 U/mL) in 200 μL of phosphate buffer pH 7.0 and then kept warm for 10 min at 37 °C in the incubator. After that, 35 μL of phosphate buffer pH 7.0-based 5 mM PNPG solution was added and reincubated for 15 min at 37 °C, and then the absorbance was calculated at the wavelength of 405 nm using a UV spectrometer. The control group used the sample in the absence of the roasted coffee extract, and the blank was the sample devoid of the alpha-glucosidase enzyme. The following formula has been computed for the percentage of alpha-glucosidase inhibition

$$\%\ \text{inhibition of alpha} - \text{glucosidase} = \left(\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}\right) \times 100\%$$

$\text{Abs}_{\text{control}}$ and $\text{Abs}_{\text{sample}}$ are the absorbances of the control and sample, respectively. The calculation of the $IC_{50}$ value was accomplished by varying the sample concentration between 0.25 and 2.00 mg/mL. The linear regression between the percentage of inhibition and the sample concentration was used to calculate the $IC_{50}$ value. The Michaelis−Menten kinetic parameters were also observed in this study. The PNPG substrate concentration was varied from 0.34 to 2.39 mM to calculate these parameters. The multiple reciprocal plots were utilized to determine the $K_M$ and $V_{max}$ values without and with the inhibitor (*I*), including the type of inhibition (competitive, noncompetitive, or mixed-type).

## STATISTICAL DATA ANALYSIS FOR BIOLOGICAL ACTIVITY

The data regarding the biological activity, including antioxidants and inhibition of alpha-glucosidase, were presented as the mean value along with the standard deviation (SD) for six replicate assessments. Duncan's multiple range test one-way analysis of variance (ANOVA) was utilized throughout the statistical examination of biological activity data that was carried out in Microsoft Excel (Microsoft Office 365). Typically, projection to latent structure (PLS) was used to determine the relationship between two data sets, *X* (predictor) and *Y* (response). In our case, the metabolomic data are the *X*-data, and the biological activity values, such as DPPH, ABTS, FRAP, and alpha-glucosidase inhibitory activity, are the *Y*-data. The purpose of this PLS modeling is to predict the chemical shifts responsible for the bioactivity of the coffee extract based on the metabolomic data. The value of biological activity used for the PLS measurement is $1/IC_{50}$. The samples that have a high level of activity are on the positive side, while the samples that have a low level of activity are on the negative side. VIP values from the PLS model were used. If the VIP value for variable *X* is greater than 1, it indicates that *X* is an important variable, whereas a value of less than 0.5 indicates that *X* is not an important variable.



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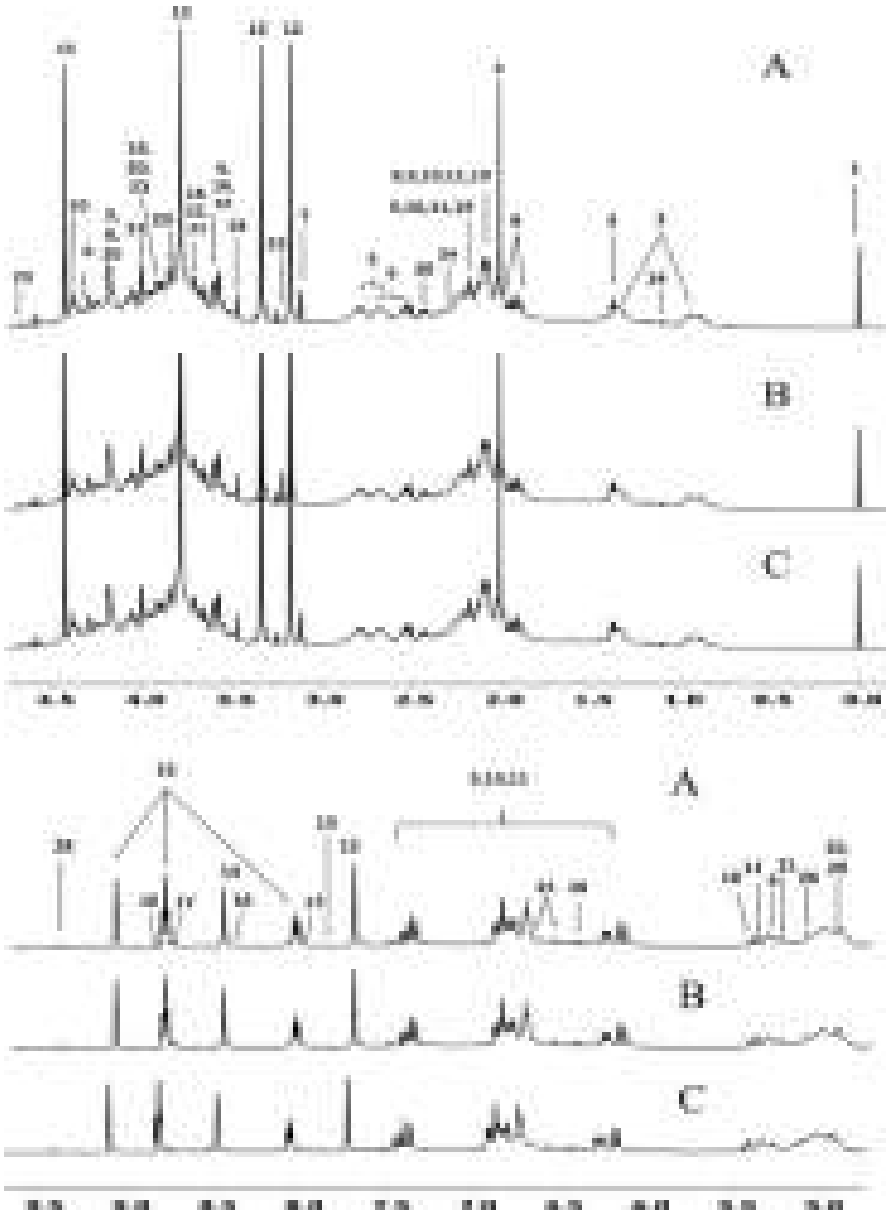


**Figure 1.** $^{1}$H NMR spectrum of the roasted coffee samples. (A) Roasted beans of regular coffee (RRA), (B) roasted beans of wild civet coffee (RWC), (C) roasted beans of caged coffee (RCC). (1) TSP, 2. lipid, (3) lactic acid, (4) acetic acid, (5) citric acid, (6) malic acid, (7) choline, (8) quinic acid, (9) 5-CQA, (10) 4-CQA, (11) 3-CQA, (12) caffeine, (13) myo-inositol, (14) formic acid, (15) trigonelline, (16) nicotinic acid, (17) *N*-methylpyridinium, (18) sucrose, (19) syllo-quinic acid, (20) $\gamma$-quinide, (21) $\alpha$-(1−3)-L-arabinofuranose (3-arabinose unit) (22) $\beta$-(1−4)-D-mannopyranose (4-mannose unit), (23) $\beta$-(1−3)-D-galacto-pyranose (3-galactose unit), (24) propionic acid, (25) catechol, (26) $\alpha$-(1−5)-L-arabinofuranose (5-arabinose unit), (27) 2-furyl methanol, (28) 5-HMF.

## RESULTS AND DISCUSSION

**Identification of Metabolites.** Identification of metabolites in the samples of roasted civet coffee (both caged and wild civet coffee) and regular coffee was accomplished by identifying their specific signals in the $^{1}$H NMR spectra, according to Figure 1. These assignments were confirmed by the 2D NMR approach, which included the TOCSY, COSY, and J-resolved spectra. The application of 2D NMR is advantageous in interpreting the signal on the $^{1}$H NMR spectrum of extracts containing multiple metabolites. The COSY and TOCSY spectra can detect proton signals originating from one molecule. Meanwhile, the J-resolved spectrum provides information regarding the coupling constant and signal multiplicity obscured by the signal overlap in the $^{1}$H NMR spectrum. The correlation of the signals of several identified metabolites on the COSY spectra is depicted in Figure 2. The identification results were further confirmed using data on the chemical shift of proton metabolites in the human metabolite database (www.hmdb.ca) and various literature related to roasted coffee metabolites.[18,28,29]

As depicted in Figure 1, common metabolites present in the green coffee beans, including sucrose, trigonelline, caffeine, quinic acid, citric acid, malic acid, acetic acid, formic acid, lipids, choline, myo-inositol, and three isomers of chlorogenic acid, namely 3-CQA, 4-CQA, and 5-CQA, were still detected in the $^{1}$H NMR spectra of all roasted coffee samples. Meanwhile, metabolites formed throughout the roasting process were also detected, namely propionic acid, sylloquinic


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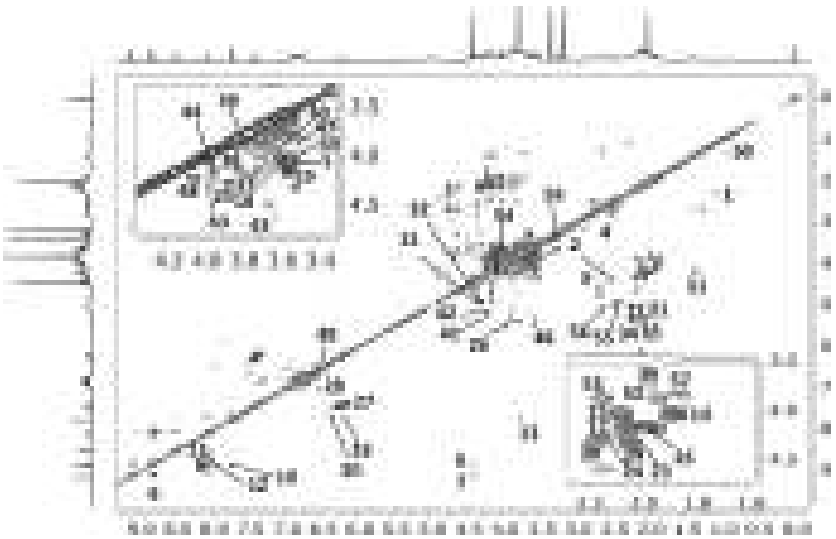

**Figure 2.** Signal correlations of characterized metabolites in the COSY spectrum. (1) H2/H3 of propionic acid, (2) H3a/H2 of malic acid, (3) H3b/H2 of malic acid, (4) H3a/H3b of malic acid, (5) H5/H4 of myo-inositol, (6) NCH3/H6 of trigonelline, (7) NCH3/H2 of trigonelline, (8) H5/H6 of trigonelline, (9) H4/H2 of trigonelline, (10) H5/H4 of nicotinic acid, (11) H3/H4 of *N*-methylpyridinium, (12) H2/H3 of *N*-methylpyridinium, (13) N7$CH_3$/H8 of caffeine, (14) H6a/H5 of quinic acid, (15) H6e/H5 of quinic acid, (16) H2a/H3 of quinic acid, (17) H2e/H3 of quinic acid, (18) H4/H5 of quinic acid, (19) H3/H4 of quinic acid, (20) H3/H4 of 2-furyl methanol, (21) H6a/H5 of 5-CQA, (22) H6e/H5 of 4-CQA, (23) H2a/H3of 5-CQA, (24) H2e/H3 of 5-CQA, (25) H3/H4 of 5-CQA, (26) H4/H5 of 5-CQA, (27) H2′/H3′ of 5-CQA, (28) H8′/H9′ of 5-CQA, (29) H6a/H5 of 4-CQA, (30) H6a/H5 of 4-CQA, (31) H3/H4 of 4-CQA, (32) H4/H5 of 4-CQA, (33) H2′/H3′ of 4-CQA, (34) H2a/H3 of 3-CQA, (35) H2e/H3 of 3-CQA, (36) H6e/H5 of 3-CQA, (37) H5/H4 of 3-CQA, (38) H2/H3 of 4-mannose unit, (39) H3/H4 of 4-mannose unit, (40) H4/H5 of 4-mannose unit, (41) H1/H2 of 3-arabinose unit, (42) H1/H2 of 5-arabinose unit, (43) H2/H3 of 5-arabinose unit, (44) H3/H4 of 5-arabinose unit, (45) H1/H3 of galactose unit, (46) H1/H2 of sucrose, (47) H2/H3 of sucrose, (48) H3′/H4′ of sucrose, (49) H1/H2 of catechol, (50) $-CH_2-CH_3/CH_2$ of lipids, (51) H2/H3 of lactate, (52) H2a/H3 of $\gamma$-quinide, (53) H2e/H3 of $\gamma$-quinide, (54) H4/H5 of $\gamma$-quinide, (55) H5/H6a of $\gamma$-quinide, (56) H5/H6e of $\gamma$-quinide, (57). H2/H3 of syllo-quinic acid, (58) NCH3/H1 of choline, (59) H1/H2 of choline.

acid, $\gamma$-quinide, $\alpha$-(1−3)-L-arabinose, $\alpha$-(1−5)-L-arabinofuranose, $\beta$-(1−4)-D-mannopyranose, $\beta$-(1−3)-D-galactopyranose, 5-hydroxy methyl furfural (5-HMF), 2-furyl methanol, *N*-methylpyridinium, nicotinic acid, and catechol. In total, 27 metabolites were identified in the aqueous extracts of roasted civet coffee (caged and wild civet coffee) and regular coffee samples.

Our previous study comprehensively discussed the metabolite elucidation in green coffee beans.[30] As a major component, sucrose signals can be readily detected in green coffee beans. In contrast to roasted coffee, 11 sucrose signals were detected in the roasted coffee sample, all of which were relatively weak signals throughout the range of 3.49−5.43 ppm, making it relatively difficult to distinguish between the signals of sucrose. The characteristic correlation between the H-2 of sucrose at $\delta$ 3.57 ppm with the anomeric H atom of sucrose at $\delta$ 5.43 ppm was described by the cross-peak 46 in Figure 2. Thus, this enhanced the assignment of the proton signals of sucrose.

Caffeine (1,3,7-trimethylxanthine) and trigonelline were naturally striking alkaloids obtained in coffee. The caffeine signals were straightforwardly identified as they are major compounds. The aromatic proton of caffeine was determined to be at $\delta$ 7.77 ppm, whereas the three *N*-methyl groups were detected as singlet signals at $\delta$ 3.88, 3.33, and 3.17 ppm, appointed as N7$CH_3$, N1$CH_3$, and N3$CH_3$, respectively. Meanwhile, four proton signals at $\delta$ 9.10, 8.86, 8.80, and 8.06 ppm in the downfield spectra region were identified as the pyridine ring of trigonelline, while the singlet signal at $\delta$ 4.43 ppm was assigned as the *N*-methyl group of trigonelline. These two doublet proton signals with large constant couplings at $\delta$ 6.28 and $\delta$ 7.51 ppm had been confirmed as the olefinic proton signals of 5-CQA, though those at $\delta$ 6.36 and $\delta$ 7.56 ppm were identified as olefinic proton signals of 4-CQA and 3-CQA, respectively. The detailed chemical shift information on chlorogenic acid isomers is described in Table 1. The methylene signals of malic acid were recognized as the double doublet signals at $\delta$ 2.46 and $\delta$ 2.71 ppm. The formic acid signal was distinctly identified at $\delta$ 8.47 ppm. Citric acid methylene groups were detected as doublet signals at $\delta$ 2.58 and 2.72 ppm. The intense singlet signal at $\delta$ 1.99 ppm was also identified as acetic acid. Furthermore, the signal of lactic acid at $\delta$ 1.31 ppm overlapped with an unidentified lipid signal, resulting in a broad signal. Myo-inositol signal was detected at $\delta$ 3.28, 3.54, 3.64, and 4.02 ppm, and a singlet signal at $\delta$ 3.21 ppm was identified as three methyl choline groups.

The presence of $\gamma$-quinide was discovered in the $^1$H NMR spectra of roasted coffee samples. This compound appears during the roasting process due to the breakdown of chlorogenic acid, as shown in Figure 3. The deshielding methine group signal at $\delta$ 4.93 ppm was assigned to $\gamma$-quinide. The proximity of the methine group to the cyclic ester group is thought to be responsible for the more deshielded methane signal. Meanwhile, the methylene signal of syllo-quinic acid was observed at $\delta$ 1.90 and 2.07 ppm. Catechol, another degraded product from chlorogenic acid, was identified at $\delta$ 6.65 and 6.70 ppm, designed as H-3 and H-4 of catechol.

Additionally, sugar compounds in roasted coffee samples, including arabinose, mannose, and galactose, were identified. The correlation of the proton signal at $\delta$ 5.25 ppm (H-1) and $\delta$ 4.25 ppm (H-2), as portrayed at cross-peak 41 in Figure 2, encouraged the assignment of 3-arabinose proton signals. The


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Table 1. Assignment of the $^1$H NMR Signals of the Identified Metabolites in all Roasted Coffee Samples[a]

| | metabolite | chemical shift (in ppm), multiplicity, coupling constants (in Hz), and number of proton | RRA | RCC | RWC |
|---|---|---|---|---|---|
| 1 | sucrose | 3.49 (t, 9.51, H-4), 3.57 (dd, 11.72, 5.03, H-2), 3.70 (s, H-1′), 3.79 (t, 9.51, H-3), 3.84 (m, H-6′/H-6), 3.88 (dd, H-5), 3.90 (dd, H-5′), 4,08 (t, 9.51, H-4′), 4.23 (d, 9.27, H-3′), 5.42 (d, 4,51, H-1) | + | + | + |
| 2 | trigonelline | 4.43 (s, $NCH_3$), 8.06 (t, H-5), 8.80 (m, H-4), 9.10 (s, H-2) | + | + | + |
| 3 | caffeine | 3.22 (s, $N3CH_3$), 3.39 (s, $N1CH_3$), 3.78 (s, $N7CH_3$), 7.77 (s, H-8) | + | + | + |
| 4 | 5-CQA (5-caffeoylquinic acid) | 2.07/2.17 (m, H-6), 2. 04/2.23 (m, H-2), 3.90 (dd, 9.9; 4.5, H-4), 4.28 (d, H-3), 5.33 (m, H-5), 6.24 (d, 16.04, H-2′), 7.38 (d, 16.04, H-3′), 6.91 (dd, 8.26; 1.63, H-5′), 6.78 (d, 8.68, H-6′), 6.87 (d, H-9′) | + | + | + |
| 5 | 4-CQA (4-caffeoylquinic acid) | 2.06/2.17 (m, H-6), 2.04/2.18 (m, H-2), 4.92 (dd, 11.18; 9, H-4), 4.36 (d, H-5), 4.30 (m, H-5), 6.26 (d, 16.04, H-2′), 7.44 (d, 16,04, H-3′), 6.91 (dd, 8.26; 1.63, H-5′), 6.78 (d, 8.68, H-6′), 6.87 (d, H-9′) | + | + | + |
| 6 | 3-CQA (3-caffeoylquinic acid) | 2.00/2.07 (m, H-6), 2.00/2.22 (m, H-2), 4.19 (dd, H-4), 5.40 (m, H-3), 3.77 (m, H-5), 6.29 (d, 16.04, H-2′), 7.44 (d, 16.04, H-3′), 6.91 (dd, 8.26; 1.63, H-5′), 6.78 (d, 8.68, H-6′), δ 6.87 (d, H-9′) | + | + | + |
| 7 | quinic acid | 1.89 (dd, H-2), 2.07 (m, H-2e/H6e), 3.55 (dd, H-4), 4.15 (m, H-5), 1.98 (dd, H-6a) | + | + | + |
| 8 | malic acid | 2.46 (dd, 15.16; 8,86, H-3), 2.70 (dd, 15.79; 8.3, H-3), 4.33 (dd, 15.55; 8.54, H-2) | | | |
| 9 | citric acid | 2.65 (d, 20.30, H-3), 2.72 (d, 22.53, H-3) | + | + | + |
| 10 | formic acid | 8.44 (d, H-1) | + | + | + |
| 11 | lactic acid | 1.36 (d, 3.29, H-3), 4.19 (q, 3.38, H-2) | + | + | + |
| 12 | lipids | 0.94 (br s, $-CH_2-CH_3$), 1.34 (br s, $(-CH2-)n$) | + | + | + |
| 13 | myo-inositol | 3.53 (dd, H1/H3), δ 4.06 (t, 5.77, H4/H6), 3.27 (t, H2), 3.62 (t, 5.80, H5) | + | + | + |
| 14 | choline | 3.21 (s, $N(CH_3)_3$), 3.50 (t, H-1), 4.04 (t, H-2) | + | + | + |
| 15 | syllo-quinic acid | 1.90 (dd, 2.97; 9.05, H-2/H-6), 2.07 (m, H-2e/H-6e), 3.36 (t, H-4), 3.83 (m, H-3/H-5) | + | + | + |
| 16 | γ-quinide | 2.00/2.17 (dd, H-2), 3.87 (m, H-3), 4.19 (dd, H-4), 4.93 (m, H-5), 2.43/2.51 (dd, H-6) | + | + | + |
| 17 | α-(1−3)-L-arabinofuranose | 5.25 (d, 3.89, H-1), 4.25 (t, 4.04, H-2), 3.90 (t, H-3), 4.15 (m, H-4), 3.64/3.75 (dd, H-5) | | | |
| 18 | α-(1−5)-L-arabinofuranose | 5.10 (d, H-1), 4.16 (t, H-2), 3.94 (t, H-3), 4.07 (m, H-4), 3.77 (dd, H-5), 3.88 (dd, H-5) | + | + | + |
| 19 | β-(1−4)-D-mannopyranose | 4.76 (d, 2.28, H-1), 4.15 (t, 2.56, H-2), 3.79 (dd, H-3), 3.93 (t, 2.54, H-4), 3.57 (m, H-5), 3.74/3.92 (dd, H-6) | + | + | + |
| 20 | β-(1−3)-D-galacto-pyranose | 4.63 (d, 5.01, H-1), 3.79 (t, H-2), 3.88 (dd, 5.29; 3.61, H-3), 4.25 (t, H-4), 3.70 (m, H-5), 3.66/3.70 (dd, H-6) | + | + | + |
| 21 | 5-HMF (5-hydroxymethylfurfural) | 7.45 (d, 16.11, H-3), 6.32 (d, 16.11, H-4), 9.44 (s, H-1), 3.62 (d, H-6), 4.58 (d, H-6) | + | + | + |
| 22 | 2-furyl methanol | 7.50 (d, H-5), 6.46 (d, H-3), 4.58 (s, H-6) | + | + | + |
| 23 | *N*-methylpyridinium | 4.36 (s, $NCH_3$), 8.77 (d, H-2/H-6), 8.02 (t, H-3/H-5), 8.51 (1H, d, H-4) | + | + | + |
| 24 | nicotinic acid | 8.99 (s, H-2), 8.44 (d, 2.33, H-4), 7.79 (1H, t, 2.99, H-5), δ 8.64 (d, 2.00, H-6) | + | + | + |
| 25 | catechol | 6.66 (d, H-3/H-6), 6.70 (d, H-4/H-5) | + | + | + |
| 26 | propionic acid | 1.08 (t, H-3), 2.27 (t, H-2) | ss | | |
| 27 | acetic acid | 1.99 (s, H-1) | + | + | + |

[a]RRA: roasted regular coffee; RCC: roasted caged civet coffee; RWC: roasted wild civet coffee. +: identified, *quantification was conducted using the signal. Multiplicity in NMR including s, d, t, dd, m. s for singlet, d for doublet, t for triplet, dd for doublet of doublets, and m for multiplet.

correlation of the proton signal at δ 5.09 ppm with δ 4.12 ppm corresponded to the 5-arabinose proton, as described in a previous study.[29] Signal correlations between δ 4.15/3.79 ppm, δ 3.79/3.93 ppm, and δ 3.93/3.57 ppm (seen on the cross-peak 38−40 in Figure 2) were recognized as correlations between H-2/H-3, H-3/H-4, and H-4/H-5 of 4-mannose.[28] Meanwhile, 3-galactose was identified from the dignity of correlated δ 4.63 and δ 3.75 ppm signals. The singlet signal at δ 9.44 ppm in the $^1$H NMR spectrum of the coffee sample was characteristic of the aldehyde proton in 5-HMF. The doublet signal with low intensity at δ 6.46 ppm resulted from H-3 of 2-furyl methanol.

Intriguingly, *N*-methyl-pyridinium and nicotinic acid, the two byproducts of trigonelline created by the roasting process,[28] were correctly identified in the $^1$H NMR spectra. Weak signals at δ 8.02, 8.51, and 8.77 ppm were allegedly due to the pyridine ring on *N*-methylpyridinium. Nicotinic acid is produced through the demethylation of trigonelline. The absence of the methyl group on the nicotinic acid pyridine ring appears to make the signal of the nicotinic acid pyridine ring slightly deshielding compared to that of *N*-methylpyridinium. Nicotinic acid pyridine ring signals were found at δ 7.73, 8.43, 8.64, and 8.99 ppm. The signal at δ 8.99 ppm was assigned to H-2 of nicotinic acid. These protons were more deshielding than H-4 and H-6 proton signals due to the electron-withdrawing effect of the nitrogen and carboxylate atoms. The summarized signal assignments of the roasted coffee samples are presented in Table 1.

**Relative Quantitative Analysis.** Some metabolites were successfully quantified in all roasted coffee samples, such as acetic acid, 2-furyl methanol, caffeine, formic acid, 5-CQA, trigonelline, quinic acid, malic acid, catechol, sucrose, and 5-HMF by the quantitative $^1$H NMR technique. The results of the quantification are depicted in Table 2. The quantified metabolites in the roasted civet coffee beans were compared with those in the regular roasted coffee. The concentrations of 2-furyl methanol, caffeine, formic acid, 5-CQA, sucrose, catechol, and 5-HMF in civet coffee significantly differed from those of regular coffee. These levels are primarily found in higher concentrations in regular coffee than the others. Meanwhile, quinic acid and malic acid were higher in wild civet coffee than in the caged civet coffee. Furthermore, acetic acid and trigonelline were distinct metabolites found in caged civet coffee.

Differences in the metabolite concentration of the roasted coffee samples (caged civet, wild civet, and regular coffee) were due to the heterogeneous metabolite concentration of green coffee beans and coffee before roasting. In a prior research


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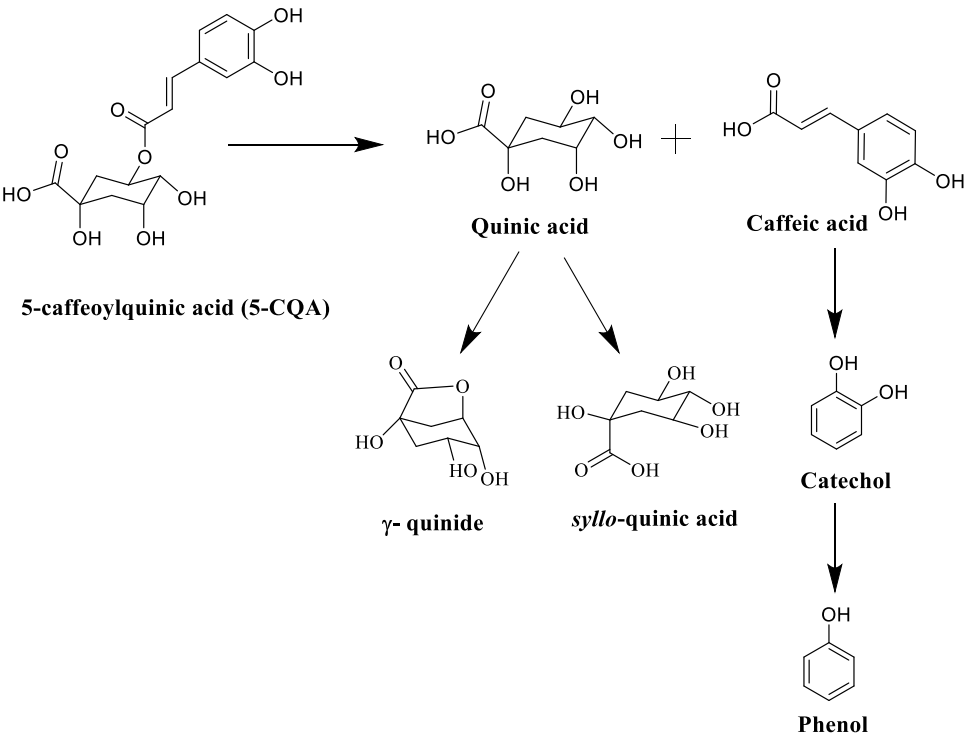


**Figure 3.** Transformation of chlorogenic acid through the roasting of coffee beans.

**Table 2. Quantified Metabolite Concentrations in all Roasted Coffee Samples**[a]

| chemical compounds | abundances in mM | | | *P* value |
|---|---|---|---|---|
| | RRA | RCC | RWC | |
| acetic acid $\delta$ 2.00 ($\delta$ 1.99−2.02) | 17.07 ± 0.24[a] | 19.87 ± 0.33[c] | 18.32 ± 0.04[b] | $1.11 \times 10^{-8}$ |
| 2-furyl methanol $\delta$ 4.58 ($\delta$ 4.57−4.59) | 2.37 ± 0.04[c] | 1.99 ± 0.03[a] | 2.06 ± 0.03[ab] | $2.11 \times 10^{-3}$ |
| caffeine $\delta$ 7.77 ($\delta$ 7.75−7.78) | 9.99 ± 0.04[c] | 6.62 ± 0.03[a] | 7.74 ± 0.03[b] | $6.11 \times 10^{-12}$ |
| formic acid $\delta$ 8.46 ($\delta$ 8.46−8.49) | 16.05 ± 0.06[c] | 14.73 ± 0.04[ab] | 13.83 ± 0.02[a] | $3.08 \times 10^{-5}$ |
| trigonelline $\delta$ 9.11 ($\delta$ 9.10−9.13) | 4.83 ± 0.05[a] | 7.14 ± 0.01[c] | 6.53 ± 0.02[b] | $2.65 \times 10^{-9}$ |
| 5-CQA $\delta$ 6.25 ($\delta$ 6.21−6.28) | 10.19 ± 0.03[c] | 8.75 ± 0.01[a] | 9.35 ± 0.08[b] | $1.00 \times 10^{-3}$ |
| sucrose $\delta$ 5.42 ($\delta$ 5.41−5.43) | 3.14 ± 0.08[c] | 2.4 ± 0.11[b] | 1.95 ± 0.09[a] | $2.30 \times 10^{-12}$ |
| quinic acid $\delta$ 1.89 ($\delta$ 1.88−1.90) | 21.65 ± 0.07[a] | 22.74 ± 0.05[b] | 24.23 ± 0.07[c] | $1.17 \times 10^{-6}$ |
| malic acid $\delta$ 4.33 ($\delta$ 4.31−4.37) | 5.55 ± 0.03[a] | 6.18 ± 0.05[b] | 8.33 ± 0.03[c] | $1.59 \times 10^{-11}$ |
| catechol $\delta$ 6.66 ($\delta$ 6.65−6.67) | 2.59 ± 0.02[c] | 2.07 ± 0.12[a] | 2.25 ± 0.07[b] | $3.99 \times 10^{-7}$ |
| 5-HMF $\delta$ 9.44 ($\delta$ 9.43−9.45) | 3.7 ± 0.04[c] | 2.24 ± 0.02[ab] | 2.07 ± 0.03[a] | $3.34 \times 10^{-8}$ |

[a]RRA: roasted regular coffee; RCC: roasted caged civet coffee; RWC: roasted wild civet coffee. The provided values represent mean ± standard deviation (SD). The repeatability of the extraction is good, as evidenced by the SD value (six repetitions) being less than 0.5. The values were derived from one-way ANOVA for each metabolite. $p < 0.05$ signifies a significant difference between the levels of each metabolite in each group. [a−c]According to Duncan's test, differences in the superscripts of the mean values for each metabolite indicate statistical significance.

involving green coffee beans (coffee beans before roasting that were used in this research), it was successfully determined that the concentration of sucrose in regular coffee (38.66 mM ± 0.16) was greater than that of wild civet coffee (31.23 mM ± 0.27).[11] Sucrose was degraded to glucose and fructose moieties during roasting. The glucose moiety was degraded to form 2-furyl methanol and organic acids, including formic acid and acetic acid. Meanwhile, the fructose moiety was converted to 5-HMF. The higher concentration of formic acid (16.05 mM ± 0.06), 2-furyl methanol (2.37 mM ± 0.04), and 5 HMF (3.70 mM ± 0.04) in the roasted regular coffee possibly caused the higher amount of sucrose in the green beans of regular coffee than the green beans of wild civet coffee, as reported in previous studies.[11] The caffeine quantity in caged civet coffee was lower (6.62 mM ± 0.03) than that in regular coffee (9.99 mM ± 0.04). According to earlier reports, the ingestion and subsequent degradation of caffeine in the small intestine of the civet into paraxanthine and methylxanthine were probably the causes of the condition. The amount of caffeine in the roasted coffee did not significantly change from that in the green coffee beans, although a portion might be lost due to sublimation.

Quinic acid levels in roasted wild civet coffee beans (24.23 mM ± 0.07) were higher than those in roasted caged civet coffee (22.74 mM ± 0.05). The formation of quinic acid in roasted coffee beans resulted from the hydrolysis of chlorogenic acid,[14] so the amount of chlorogenic acid present in green coffee beans is an important factor to consider. The green coffee beans of wild civet coffee have more chlorogenic acid than the wild ones. It may lead to a higher quinic acid content in the roasted beans of wild civet coffee than that in


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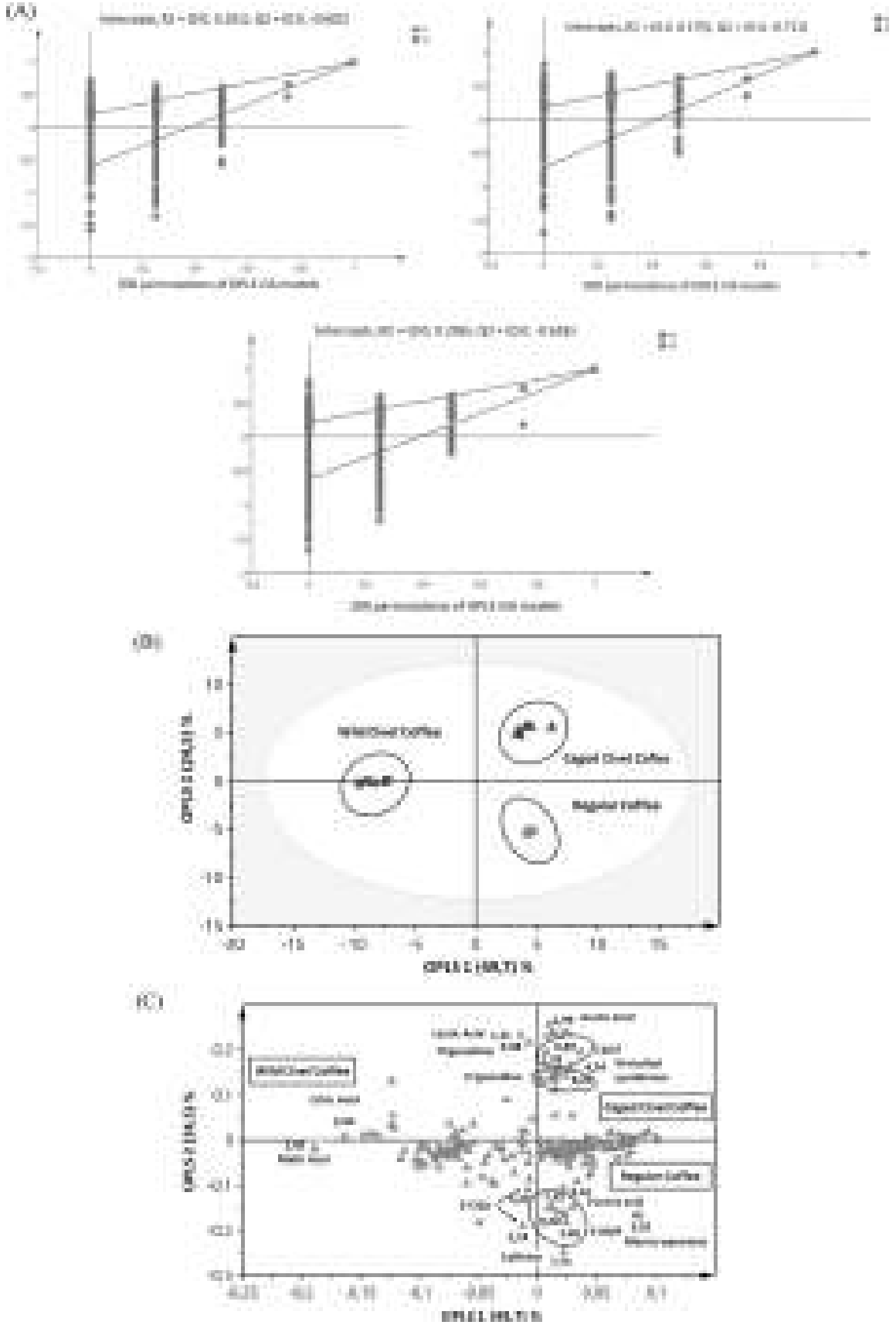

**Figure 4.** OPLSDA model plot was processed using data from all samples of roasted coffee beans. The permutation test plot showed that the OPLSDA model was correct.



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caged civet coffee. The different metabolite concentrations in the roasted coffee beans suggested that the samples contained qualitatively identical metabolites but different quantitatively. Besides that, the compound levels in the green beans affected the metabolite concentrations in the roasted coffee sample. The results of the relative quantitative analysis of the coffee samples are abridged in Table 2.

**Multivariate Data Analysis.** In order to obtain a comprehensive analysis of dissimilarities in metabolite levels within all roasted coffee samples, the entire data set from the $^1$H NMR spectra was extracted, followed by assessment using multivariate statistical analysis. OPLS-DA was utilized as a model to examine the metabolite profiles of all roasted coffee samples. The main vantage of OPLS-DA modeling lies in the relief of interpretation, remarkably in the case of multiple classes.[31] The statistical parameter OPLS-DA had total variations of 0.92 (R2X) and 0.99 (R2Y). Meanwhile, the obtained OPLS-DA model had a Q2 value of 0.98, indicating an excellent predictive level. Furthermore, this OPLS-DA model underwent a 200 permutation test for further validation (Figure 4a). As a result, the regression lines of Q2 crossed the *y*-axis at a point less than zero, which proved that the model was accurate.

The sore plot of the OPLS-DA model provided three clearly defined clusters correlating to the type of roasted coffee samples by combining the first (49.7%) and second (24.5%) components, as seen in Figure 4b. It suggested that each coffee sample had a distinct metabolite profile. Meanwhile, the loading plot showed compounds impacting the variation of the samples. As shown in the loading plot (Figure 4c), 5-HMF (buckets at $\delta$ 3.60, 7.45, and 9.40 ppm), caffeine (bucket at $\delta$ 7.75 ppm), 5-CQA (buckets at $\delta$ 2.19 and 5.29 ppm), formic acid (bucket at $\delta$ 8.42 ppm) and 4-mannose unit (bucket at $\delta$ 3.91 ppm), trigonelline (buckets at $\delta$ 4.40, 8.03, and 9.08 ppm), acetic acid (bucket at $\delta$ 1.99 ppm), lactic acid (bucket at $\delta$ 1.31 ppm), lipid (at $\delta$ 0.89 and 1.28 ppm), *N*-methyl-pyridinium (buckets at $\delta$ 4.34 and 8.79 ppm), citric acid (bucket at $\delta$ 2.64 ppm), and malic acid (bucket at $\delta$ 2.45 ppm) were discovered to contribute to the classification of roasted coffee samples.

For a better understanding of distinguishing metabolites for each sample of a roasted coffee sample, three models of two-class OPLS-DA were constructed. *S*-Plot was applied for the visualization of these models. The *S*-plot allows for identifying potentially significant variables by combining the modeled correlation (*Y*-axis) and covariance (*X*-axis) from the OPLS-DA scatter plot. The metabolite with the highest *p* and *p*(corr) values is considered the most important for classifying samples. The first of the two-class models was engendered to distinguish caged civet coffee from regular coffee. This model yielded an R2X value of 0.81, an R2Y value of 0.99, and a Q2 value of 0.99. The conforming *S*-plot (Figure 5a) showed that the metabolite of caged civet coffee was distinguished by trigonelline (buckets at $\delta$ 4.40, 8.03, and 9.08 ppm), lipid (buckets at $\delta$ 0.89 and 1.28 ppm), acetic acid (bucket at $\delta$ 1.99 ppm), and lactic acid (bucket $\delta$ 1.31 ppm). Meanwhile, regular coffee was recognized with a 4-mannose unit (buckets at $\delta$ 3.72 and 3.91 ppm), 5-HMF (buckets at $\delta$ 3.60, 7.43, and 9.40 ppm), 5-CQA (buckets at $\delta$ 2.13, 5.29, 6.25, and 6.90 ppm), caffeine (bucket at $\delta$ 7.75 ppm), and formic acid (bucket at $\delta$ 8.42 ppm).

The next of the two-class models is acquired from the roasted beans of wild civet coffee and regular coffee. This

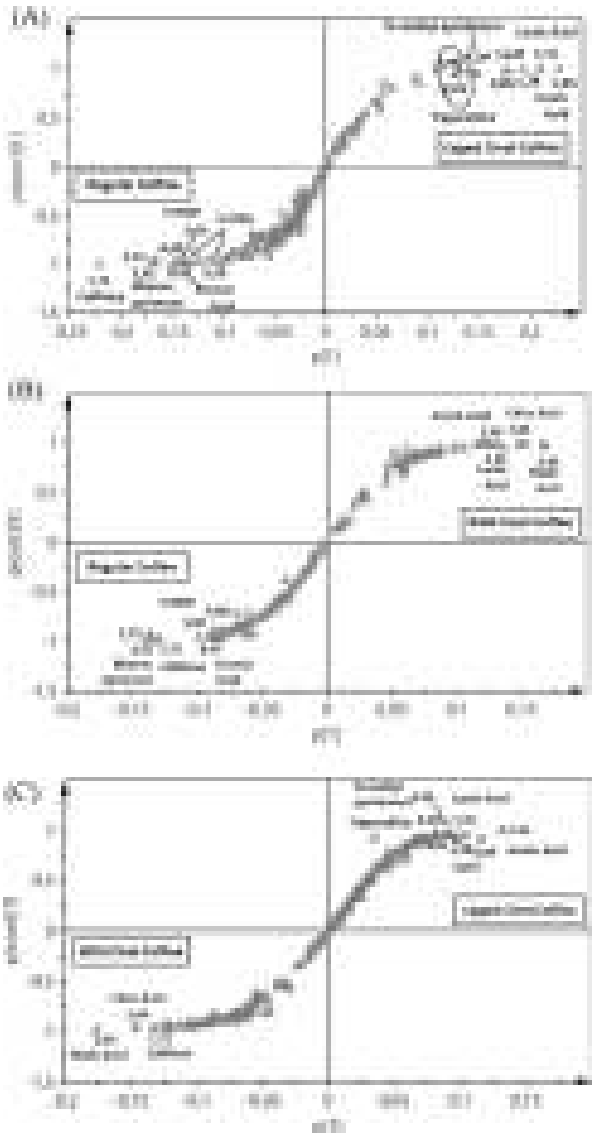

**Figure 5.** *S*-Plots produced by the two-class OPLSDA models. (A) Roasted regular coffee and roasted caged civet coffee. (B) Roasted regular coffee and roasted wild civet coffee. (C). Roasted wild civet coffee and roasted caged civet coffee.

model produced R2X, R2Y, and Q2 values of 0.79, 0.99, and 0.99, respectively. According to the corresponding *S*-plot (Figure 5b), the metabolite of wild civet coffee was characterized by citric acid (bucket at $\delta$ 2.64 ppm), lactic acid (bucket $\delta$ 1.31 ppm), and malic acid (bucket at $\delta$ 2.45 ppm). Meanwhile, formic acid (bucket at $\delta$ 8.42 ppm), caffeine (bucket at $\delta$ 7.75 pm), 5-HMF (buckets at $\delta$ 3.60 and 9.40 ppm), and 4-mannose unit (buckets at $\delta$ 3.72 and 3.91 ppm) were discovered as the distinctive metabolites in regular coffee. The last model compared the roasted coffee beans of wild civet coffee and caged civet coffee. This model emitted an R2X value of 0.88, an R2Y value of 0.99, and a Q2 value of 0.98. In this *S*-plot, caffeine (bucket at $\delta$ 7.75 ppm), citric acid (bucket at $\delta$ 2.64 ppm), and malic acid (bucket at $\delta$ 2.45 ppm) were the responsible metabolites in wild civet coffee. Meanwhile, the discriminant metabolites for the roasted beans of caged civet coffee were acetic acid (bucket at $\delta$ 1.99 ppm), lactic acid (bucket at $\delta$ 1.31 ppm), trigonelline (buckets at $\delta$ 4.40, 8.85, and 9.09 ppm), *N*-methyl-pyridinium (buckets at $\delta$ 4.34 and



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8.79 ppm) and lipid (buckets at $\delta$ 0.89 and 1.28 ppm), as presented in Figure 5c.

The OPLS-DA *S*-plot model suggests that malic acid and citric acid were the distinguishing metabolites in the roasted beans of wild civet coffee. Both citric acid and malic acid are naturally present in green coffee beans. The concentration of these compounds decreased during the roasting process, as described in prior studies.[28] However, the roasted beans of wild civet coffee still had a higher concentration of citric acid and malic acid than the other coffee samples, as presented in Table 2. The high concentrations of malic acid and citric acid in wild civet coffee were possibly due to a combination of factors. The first is the sucrose degradation into organic acids, including malic acid and citric acid, by gastric acid and/or digestive enzymes. It was verified by increasing the malic acid and citric acid levels in green coffee, followed by decreasing sucrose levels. Second, the increased concentration of malic acid and citric acid may also result from fermentation by the civet gut microbiota. Malic acid and citric acid as fermentation products possibly were absorbed into the coffee beans while in the digestive tract. Similar cases supported it, including the increase of malic acid levels in fermented tempoyak[32] and the process of malic acid formation by the microbiota.[33]

The discriminant metabolites in the roasted beans of caged civet coffee, according to the OPLS-DA *S*-plot data, were acetic acid, lactic acid, trigonelline, and lipids. Acetic acid as a characteristic metabolite in the roasted beans of caged civet coffee was confirmed in the results of the quantitative analysis described earlier. The number of lactic acid bacteria was possibly thought to affect the lactic acid levels in civet coffee. Lactic acid bacteria in the digestive system in caged civet were higher than in wild civet.[34] It was probably responsible for the higher lactic acid content in caged civet coffee than that in wild civet coffee. Meanwhile, formic acid, caffeine, 4-mannose units, and 5-HMF were the characteristic compounds in regular roasted coffee in the corresponding OPLS-DA *S*-plots. The concentration of 4-mannose was greater in regular coffee than that in civet coffee. It possibly resulted from converting 4-mannose into acetic acid and propionic acid, as explained in a previous report.[35] Formic acid and 5-HMF levels increased after the roasting process due to the degradation of sucrose during the roasting process, as described in the literature.[36]

**Antioxidant Assay.** Based on the OPLS-DA loading plot data and quantitative analysis explained above, the metabolite profiles of roasted civet coffee were confirmed to be distinct from those of regular coffee. These differences were suggested to affect its antioxidant activity. In this work, the antioxidant activity of all roasted coffee was evaluated using the DPPH, ABTS, and FRAP methods. The measurement of the antioxidant assay using the FRAP method cannot detect antioxidant properties with a mechanism of action through hydrogen atom transfer (HAT). This method was based on the ability to reduce $Fe^{3+}$ to $Fe^{2+}$ in a coffee sample. Antioxidant activity test using $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ was measured based on the ability to scavenge radicals. The ability of antioxidant compounds in coffee samples to transfer hydrogen atoms (HAT) was used to explain the mechanism of action of the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ antioxidants. The $DPPH^{\bullet}$ radical is a naturally formed radical, while the $ABTS^{\bullet+}$ cation radical is a radical formed due to the reaction between $ABTS^{\bullet+}$ and potassium persulfate. Thus, the three methods of the antioxidant assay complement each other.

The antioxidant activity of roasted beans of coffee samples was evaluated with $IC_{50}$ values on $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ and $EC_{50}$ on FRAP. The $IC_{50}$ value indicates the sample concentration obliged to inhibit 50% of the $DPPH^{\bullet}$ radicals and $ABTS^{\bullet+}$ cation radicals, while $EC_{50}$ in the FRAP capacity signifies the sample concentration needed to exhibit 50% of the FRAP capacity. The lower the $IC_{50}$ and $EC_{50}$ values, the higher is the antioxidant activity suggested. The results revealed that the higher antioxidant activity was presented by the roasted beans of wild civet coffee tested using the $DPPH^{\bullet}$ method ($IC_{50}$ = 26.69 ± 0.29 mg/L, *p*-value < 0.5), $ABTS^{\bullet+}$ method ($IC_{50}$ = 29.59 ± 0.08 mg/L, *p*-value < 0.5), and FRAP assay ($EC_{50}$ = 21.47 ± 0.23 mg/L, *p*-value < 0.5), as depicted in Table S1.1−S1.3. Meanwhile, the antioxidant activity of caged civet coffee ($IC_{50}$ = 27.42 ± 0.37 mg/L, *p*-values < 0.5) and regular coffee ($IC_{50}$ = 31.63 ± 0.03 mg/L, *p*-value < 0.5) was lower than that of roasted beans of wild civet coffee.

Antioxidant activity data were then correlated with the bucket data of the NMR spectrum using the PLS method. The aim of PLS modeling is to predict chemical shifts from the metabolomic data that are responsible for the antioxidant activity. To gain a better understanding, three PLS models were developed. The first model demonstrated a correlation between NMR data with antioxidants, as measured by the DPPH method, the correlation with the ABTS method as the second model, and the last model depicted a correlation of NMR data with the FRAP test. The PLS statistical parameters of the three models were R2X > 0.76, R2Y > 0.81, and Q2 > 0.72, indicating good predictability. A permutation test was then used to validate the PLS models (Figure S2). The permutations demonstrated that the PLS models satisfy the validity requirements.

A closer surveillance of the PLS biplot (seen in Figure S3) presented many of the above metabolite resonances positively correlated to the antioxidant activity, such as malic acid, citric acid, chlorogenic acid, and quinic acid. These data are also strengthened from the loading coefficient plot, which can be seen in Figure S4. Based on the loading coefficient plot data, the metabolites mentioned above are positively correlated with their antioxidant activity. In order to create a prioritized list of signals, we select the VIP scores (variable importance in the projections) for each positively correlated signal. The VIP is the weighted sum of squares of PLS weights.[37] The VIP PLS plot is depicted in Figure S5. In the first model, the VIP values were as follows: 2.53 for citric acid ($\delta$ 2.64 ppm), 0.62 for chlorogenic acid ($\delta$ 5.33 ppm), 2.80 for quinic acid ($\delta$ 3.54 ppm), 0.29 for trigonelline ($\delta$ 8.03 ppm), and 0.47 for caffeine ($\delta$ 7.77 ppm). Meanwhile, the VIP values of the second model were 2.52 for citric acid, 0.67 for chlorogenic acid, 2.10 for quinic acid, 0.27 for trigonelline, and 0.45 for caffeine. Furthermore, the VIP values in the last models were as follows: citric acid had 2.50, and caffeine and trigonelline had lower VIP values of 0.42, and 0.21, respectively. A tabular representation of the VIP value is provided in Table S2. Even a further signal at $\delta$ 2.45 was identified as malic acid which displayed the highest VIP values (VIP values > 3.00), tested using DPPH, ABTS, and FRAP methods. The higher the VIP value, more is the predictor that is responsible for the biological activity.[37] On the basis of the surveillance above, it can be suggested that various metabolites in coffee samples contribute to the antioxidant activity. However, malic acid plays a crucial role in the antioxidant activity of coffee.



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According to the result of a chemometric correlation, malic acid was the highest positive correlated to the antioxidant activity of the coffee sample. This result was further verified by comparing the DPPH• radical-scavenging activities of caffeine and malic acid. Caffeine as a metabolite had a low correlation with the antioxidant activity of the whole coffee sample. The $IC_{50}$ value of DPPH• radical scavenging by malic acid ($IC_{50}$ = 6.29 mg/L ± 0.55) was lower than the $IC_{50}$ value of caffeine ($IC_{50}$ = 18.26 mg/L ± 0.11). It suggests that the scavenging activity of malic acid is better than that of caffeine. The good ability of malic acid to scavenge DPPH• radicals might lead to a positive correlation between malic acid and the antioxidant activity of coffee samples. Conceivably, the higher the concentration of malic acid, the greater the antioxidant activity of the coffee samples is. Wild civet coffee contains more malic acid than the other samples explained in the quantitative analysis. Moreover, malic acid was a characteristic metabolite in the roasted beans of wild civet coffee according to the OPLS-DA data. Meanwhile, wild civet coffee had the greatest antioxidant activity compared with the other coffee samples. It validates the PLS analysis data, which showed that malic acid positively correlates with the roasted coffee antioxidant activity. Furthermore, it can be demonstrated that fermenting civet coffee into more malic acid benefits the antioxidant activity.

**Alpha-Glucosidase Inhibitory Activity.** This work assessed the ability of roasted coffee beans to inhibit alpha-glucosidase activity. Alpha-glucosidase is an enzyme found in the brush border (microvilli) of the small intestine. Inhibiting alpha-glucosidase hinders the production of sugars from carbohydrate metabolism, thereby preventing postprandial hyperglycemia, the leading cause of chronic diabetes complications.[23] Alpha-glucosidase inhibition of roasted beans of the coffee samples was expressed as the $IC_{50}$ values. Under test conditions, the $IC_{50}$ value indicates a concentration that can inhibit 50% of the alpha-glucosidase activity. The lower $IC_{50}$ value suggested higher alpha-glucosidase inhibitory activity. The results of the assay revealed that the roasted beans of wild civet coffee ($IC_{50}$ = 2.08 mg/mL ± 0.06) acquired higher inhibitions of alpha-glucosidase activity than caged civet coffee ($IC_{50}$ = 2.69 mg/mL ± 0.13) and regular coffee ($IC_{50}$ = 3.05 mg/mL ± 0.06), as depicted in Table S3.

The data of alpha-glucosidase inhibition activity were correlated with the bucket data in the NMR spectrum using PLS analysis to determine which coffee metabolite is responsible for its alpha-glucosidase inhibitory activity. The PLS statistical parameters, Q2 = 0.97, R2X = 0.78, and R2Y = 0.98, all point to a good level of predictability. The PLS models were then validated using a permutation test (see Figure S2). Through the use of permutations, it was shown that the PLS models are valid. A more in-depth observation of the PLS biplot (which can be found in Figure S3) revealed that many of the resonances of the aforementioned metabolites were positively correlated to the alpha-glucosidation inhibitory activity. These metabolites included malic acid, citric acid, chlorogenic acid, quinic acid, nicotinic acid, and trigonelline. To generate a ranked list of signals, we pick those with the highest VIP scores. The VIP values were 3.03 for malic acid ($\delta$ 2.45), 2.29 for citric acid ($\delta$ 2.64 ppm), 0.78 for chlorogenic acid ($\delta$ 5.33 ppm), 2.80 for quinic acid ($\delta$ 3.54 ppm), 0.17 for trigonelline ($\delta$ 8.80 ppm), and 0.47 for caffeine ($\delta$ 7.77 ppm). The listed VIP values of the coffee samples are shown in Table S2.

According to the PLS analysis, malic acid had the highest positive correlation with the alpha-glucosidase inhibitory activity. Further verification of this result was carried out by contrasting the levels of inhibition of alpha-glucosidase activity exhibited by malic acid and caffeine. The correlation between caffeine and inhibition of the alpha-glucosidase activity of the entire coffee sample was weak. Malic acid had a lower $IC_{50}$ value of the alpha-glucosidase inhibitory activity ($IC_{50}$ = 0.24 mg/mL ± 0.02) than caffeine ($IC_{50}$ = 1.09 mg/mL ± 0.08). It indicates that malic acid possessed effective alpha-glucosidase inhibiting properties. These results aligned with those reported in earlier studies.[27] Interestingly, wild civet coffee showed the best alpha-glucosidase inhibition activity than the other coffee samples and had higher malic acid levels than the others. It was reflected in the quantitative data and statistical analysis of OPLSDA. The possibility of coffee fermentation in the civet digestive tract increases malic acid levels so that it has a positive effect on its alpha-glucosidase inhibitory activity.

The inhibition kinetics of the roasted beans' extract of wild civet coffee as the highest inhibitory activity was evaluated with the Lineweaver−Burk plot analysis. The data from the regression analysis showed convergent lines that intersected the *y*-axis and the *x*-axis. The R2 value for this analysis was less than 0.97, as shown in Figure S6. The results indicated that the mechanism of alpha-glucosidase inhibition by coffee samples was carried out with mixed inhibition mechanisms. It can be seen from the ability of the coffee samples to decrease the $V_{max}$ value and increase the $K_M$ value, as represented in Table S4. It was consistent with earlier studies regarding the coffee sample-mixed inhibition mechanism studies.[22] Intriguingly, according to the literature study, malic acid has a noncompetitive inhibitory mechanism.[27] It was distinct from the coffee samples with a mixed mechanism of inhibition. In spite of this, it suggested that malic acid was not the only compound contributing to the alpha-glucosidase inhibitory activity of the coffee samples. Trigonelline, quinic acid, 5-CQA, and the other metabolites all contributed to the activity, but regrettably, to the best of our knowledge, no scientific data have been published on their inhibition processes.

Finally, the metabolite profiles of the roasted beans of civet coffee samples have been analyzed with $^1$H NMR-based metabolomics. In total, 27 compounds in the roasted coffee samples were successfully identified. The results showed that civet coffee and regular arabica coffee were qualitatively similar. However, there were differences in the metabolite composition in each coffee sample. OPLS-DA was applied to reveal the similarities and differences within the coffee samples. The results showed that roasted civet coffee had higher levels of acetic acid, trigonelline, quinic acid, citric acid, and malic acid than regular coffee. Antioxidant properties and alpha-glucosidase activity were evaluated in all coffee samples. This is the first scientific report on the antioxidant and alpha-glucosidase inhibitory properties of wild and caged civet coffee compared to regular coffee. Both of these tests are related to in vitro antidiabetic testing. Antioxidants can reduce oxidative damage that causes diabetes,[21] while alpha-glucosidase inhibition can reduce the risk of diabetes by controlling sugar formation.[38] Though very early, this study's antioxidant activity and alpha-glucosidase testing indicate that luwak coffee has greater antidiabetic properties than regular coffee.


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## ■ ASSOCIATED CONTENT

### Supporting Information

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$IC_{50}$ values for the antioxidant activity of all roasted coffee samples; VIP values; $IC_{50}$ values of the alpha-glucosidase inhibitory activity of roasted coffee samples; alpha-glucosidase activity kinetic parameters of the coffee extracts; summary of the sampling method; permutation of the PLS models; PLS biplot of the coffee samples; loading coefficient PLS plot of the coffee samples; VIP PLS plot; and reciprocal Lineweaver−Burk plot with and without the coffee extract (PDF)

## ■ AUTHOR INFORMATION


### Corresponding Author

**Nizar Happyana** − *Organic Chemistry Division, Faculty of Mathematics and Natural Sciences, Bandung Institute of Technology, Bandung, West Java 40132, Indonesia;* orcid.org/0000-0002-3521-0855; Phone: +62222502103; Email: nizar@chem.itb.ac.id; Fax: +62222504154

### Authors

**Lizma Febrina** − *Organic Chemistry Division, Faculty of Mathematics and Natural Sciences, Bandung Institute of Technology, Bandung, West Java 40132, Indonesia; Pharmaceuticals Research and Development Laboratory of Farmaka Tropis, Faculty of Pharmacy, Mulawarman University, Samarinda, East Kalimantan 75119, Indonesia*

**Yana Maolana Syah** − *Organic Chemistry Division, Faculty of Mathematics and Natural Sciences, Bandung Institute of Technology, Bandung, West Java 40132, Indonesia*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsfoodscitech.3c00249


### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

$^1$H NMR, proton nuclear magnetic resonance; COSY, correlated spectroscopy; TOCSY, total correlation spectroscopy; OPLS-DA, orthogonal projection to latent structure-discriminant analysis; PLS, partial least-squares; DPPH, 2,2-diphenyl-1-picrylhydrazil; ABTS (2, 2-azino-bis(3-ethyl benzothiazoline-6-sulfonic acid); TPTZ, tris-pyridyltriazine; PNPG, 4-nitrophenyl-beta-D-glucopyranoside; ANOVA, analysis of variance; $IC_{50}$, half-maximal inhibitory concentration; $EC_{50}$, half-maximal exhibit concentration; $K_M$, Michaelis constant; $V_{maks}$, maximum velocity; CQA, caffeoylquinic acid

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